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# ANIMAL REPRODUCTION

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**Proceedings of the 27th Annual Meeting of the Brazilian Embryo Technology Society (SBTE),  
August 29th to September 1st, 2013, Praia do Forte, BA, Brazil.**

From the President

Dear Colleagues,

We are pleased to announce the Proceedings of the 27th Annual Meeting of the Brazilian Embryo Technology Society (SBTE) from August 29th to September 1st in Praia do Forte, BA. In the 27th SBTE Meeting our main aspiration is to maintain the scientific quality that we have all become used to. As in 2012, our proceedings will be published in the international journal *Animal Reproduction*, considered the most relevant Latin American scientific journal in our field. We would like to thank the extraordinary support from the Editors and staff of this Journal and to the Brazilian College of Animal Reproduction (CBRA) to embrace this collaboration with SBTE. The current SBTE meeting is once again a joint event with the Brazilian Artificial Insemination Society (ASBIA) and the South American Research Consortium on Cloning and Transgenesis in Ruminants. As an innovation, we introduced this year a new workshop on In Vitro Embryo Production, executed in collaboration with Brazilian IVF companies. Brazil is a leader in the IVF embryo industry, producing 70% of all IVF embryos in the world. We strongly believe in the contribution of this workshop to the in vitro embryo production industry and to the continuous development of this technology. We understand that promotion of combined events consists of a great opportunity for intellectual collaboration among scientists, researchers, professionals, and students from Brazil and abroad. We will maintain the lecture session structure of the 2012 event. We believe that a very positive change was implemented in the scientific program last year. All plenary sessions will be followed by concurrent scientific and applied presentations related to the same topics. These combined presentations make it possible for the attendees to follow specific aspects of the theme, according to professional interest. We would like to thank CAPES, CNPq, and FAPESP, as well as the pharmaceutical and veterinary equipment industry and farmers that support our event. Without their generosity it would not have been possible to make our meeting come true. We will be pleased to have you at our event. We sincerely hope that all those attending will enjoy the 27th Annual Meeting of SBTE in Praia do Forte, BA, Brazil.

**Pietro S. Baruselli**  
President of the SBTE  
(2012-2013)



**Proceedings of the 27th Annual Meeting of the Brazilian Embryo Technology Society (SBTE),  
August 29th to September 1st, 2013, Praia do Forte, BA, Brazil.**

From the Scientific Committee Chair

Greetings and welcome to the 27th SBTE Annual Meeting! The Scientific Committee was very happy with the positive feedback received from the members of the society after last year's meeting in Foz do Iguaçu. Therefore, we maintained the program structure for this year. Again, we invited world-class speakers to lecture about up-to-date information on embryo technology-related fields. The main program was divided in four sessions, one for each of the main biological components involved in embryo technology: the ovary, the uterus, the gametes, and the embryo itself. In the first half of each session, speakers were carefully picked to deliver contents of general interest to the society. In the second half, attendees will choose between two concurrent sessions, one focused on more basic aspects ("SBTE science") and the other emphasizing the more applied aspects ("SBTE technology") of that session's topic. Special emphasis was directed to the pre-conference workshops. An exciting, brand new workshop on In Vitro Embryo Production was introduced in this year's meeting, to provide a forum for new information and discussions on this crucial topic on Brazilian embryo industry. The equine workshop was extended and renewed to attract more of the horse reproduction community. Last but not least, the second editions of the ASBIA workshop, on artificial insemination, and the PROSUL workshop, on transgenesis will again highlight progress in these fields of research. The SBTE wants to thank all members that sent their best work to be presented at this meeting. The real value of SBTE is the scientific and technologic progress it brings to the Brazilian society. Furthermore, SBTE wants to acknowledge the speakers, for putting a lot of effort on the preparation of excellent manuscripts and lectures to be delivered at this year's meeting. Undoubtedly, considering the breadth and depth of the information presented in this volume, it is due to become an international reference for years to come. Finally, there is a long list of people to whom the making of the SBTE proceedings was a high priority for the last few months and that SBTE needs to thank enthusiastically. This list includes all folks from the SBTE administrative board, abstract session coordinators, abstract reviewers, manuscript reviewers, and scientific editors. Once again, SBTE thanks the editors and staff at the Animal Reproduction journal and the Colégio Brasileiro de Reprodução Animal, for their collaborative spirit and instrumental help on putting together this year's meeting proceedings. We hope you find this volume informative and useful. See you in Praia do Forte!

**Mario Binelli, PhD**

Chairman of the SBTE Scientific Committee  
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**Proceedings of the 27th Annual Meeting of the Brazilian Embryo Technology Society (SBTE),  
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## Synchronization techniques to increase the utilization of artificial insemination in beef and dairy cattle

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### Abstract

The main objective of the implementation of Artificial Insemination (AI) in cattle is to produce a sustained genetic progress in the herd. Although AI is an old reproductive biotechnology, its widespread implementation is very recent and is mainly due to the use of protocols that allows the AI without heat detection, commonly called fixed-time artificial insemination (FTAI). The development of FTAI protocols also allowed the application of AI in larger, extensively managed, herds and especially in suckled cows instead of just reducing the breeding programs to the heifers. FTAI treatments are widely used in South America, with about 2,500,000 cows inseminated in the last season in Argentina and about 6,500,000 in Brazil. This manuscript aims to present and describe several treatments available and some of the factors that may affect pregnancy rates.

**Keywords:** eCG, estradiol, fixed-time AI, GnRH, progestins.

### Introduction

Artificial Insemination has been used widely to reproduce the most valuable genetics. However, factors such as nutrition, management, and estrus detection efficiency affect the widespread use of this technology in most cattle operations. The most useful alternative to increase the number cows inseminated is to apply protocols that allow for AI without the need for estrus detection, usually referred to as fixed-time AI (FTAI). The objective of this manuscript is to review the available protocols that synchronize ovulation in beef and dairy cattle, making a special emphasis in those currently used in South-America.

### Synchronization treatments in beef cattle

Estradiol and progestin treatments have been widely used over the past several years in estrus synchronization programs in cattle (Macmillan and Burke, 1996; Bó and Baruselli, 2002) and are the preferred treatment for FTAI of beef cattle in South

America. Treatments consist of insertion of a progestin-releasing device and the administration of estradiol on day 0 (to synchronize follicular wave emergence), PGF at the time of device removal on days 7, 8 or 9 (to ensure luteolysis), and the subsequent application of a lower dose of estradiol 24 h later or GnRH/LH 48 to 54 h later to synchronize ovulation (Bó *et al.*, 2002a, b; Martinez *et al.*, 2002a). The pregnancy per AI (P/AI) reported with these protocols has been between 40 to 50%, ranging from 27.8 to 75.0%. The factors that most affected pregnancy rates were body condition score (BCS) and cyclicity of the cows (Bó *et al.*, 2002b; 2007).

The application of equine Chorionic Gonadotropin (eCG) at the time of removal of a progestin device has been extensively used in *Bos indicus* herds (reviewed in Baruselli *et al.*, 2004) and in *Bos taurus* herds with high incidence of postpartum anestrus (Bó *et al.*, 2002b). Probably the most important effect of eCG is the stimulation of the growth of the dominant follicle that consequently increases ovulation rate (Sá Filho *et al.*, 2010), especially in cows in postpartum anestrus and/or in low BCS (Bó *et al.*, 2002b; 2007). Analysis from 9,668 FTAI has shown that animals treated with progestin-devices must have a BCS higher than 2.5 (scale 1 to 5) and ideally >3 to achieve pregnancy rates of 50% or higher (Bó *et al.*, 2007). Conversely, the addition of eCG allowed for pregnancy rates close to 50% in cows with a BCS of ≤2.5 (Bó *et al.*, 2007). It is very important to note that these results have been achieved only when cows were gaining body condition during the breeding season. If drought conditions or lack of feed prevent cattle from improving body condition during the breeding season, pregnancy rates will most probably be 35% or less, even after the administration of eCG (Bó *et al.*, 2007).

Restricted suckling or calf removal associated with progestin devices has also been used for the induction of cyclicity in beef cows (Williams *et al.*, 2002). However the response to temporary weaning (TW) seems to be related to body condition of the cows. Two experiments were conducted to compare the effects of eCG treatment and TW on ovulation and pregnancy rates in postpartum cows in moderate to low body condition and only 22% of them with a CL (Bó *et al.*,

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2007). We found that both TW and eCG increased ovulation rates [(TW = 7/10, 70.0%; eCG = 12/20, 60.0%; and control cows (no TW or eCG treatment: 2/9, 22.2%)]. Although the growth rate of the ovulatory follicle was greater ( $P < 0.05$ ) in cows treated with eCG ( $1.1 \pm 0.1$  mm/day) than in those not treated with eCG ( $0.6 \pm 0.1$  mm/day), the ovulatory follicle was smaller in TW cows ( $9.9 \pm 0.4$  mm), compared to those not TW ( $11.8 \pm 0.3$  mm;  $P < 0.05$ ). P/AI were increased in cows treated with eCG (eCG, 154/377, 40.8% vs. no eCG, 128/392, 32.6%;  $P < 0.01$ ); whereas no differences were found between cows that were TW (141/379, 37.2%) and those that were not (141/390, 36.1%;  $P > 0.7$ ). There was an interaction between TW and pregnancy rates that was due to an improvement in P/AI when cows had  $>2.5$  BCS (TW: 48.3% vs. no TW: 28.2;  $P < 0.05$ ) and not improvement when cows were  $\leq 2.5$  BCS (36.4 vs. 37.9%). It was concluded that the use of eCG but not TW improved P/AI following FTAI in postpartum *Bos indicus* x *Bos taurus* crossbred cows in moderate to low body condition. Results also suggest that the eCG-related increase in P/AI may be due to the final growth rate of the ovulatory follicle. On the other hand, the absence or little effect of TW on pregnancy rates contrasts with data from other studies done with Nelore cows (Penteado *et al.*, 2004; Sá Filho *et al.*, 2009). Therefore, the beneficial effects of TW may differ, depending on the management and BCS of the cows. Moreover, to set up a TW program creates logistical problems in several farms, especially in medium to small farms. Nevertheless, the results from both studies confirmed those reported previously that eCG increased P/AI in suckled anestrous cows enrolled in a FTAI program utilizing progesterin devices and estradiol (Bó *et al.*, 2002b, 2007; Baruselli *et al.*, 2004).

GnRH-based treatment protocols have been used in North-America for FTAI in beef cattle (Geary *et al.*, 2001). These treatments have also been used in FTAI programs in *Bos indicus* cattle (Barros *et al.*, 2000; Williams *et al.*, 2002; Baruselli *et al.*, 2004). However, overall pregnancy rates (Baruselli *et al.*, 2004; Saldarriaga *et al.*, 2005) have often been lower than those rates reported in *Bos taurus* cattle, with low conception rates in anestrous cows (Fernandes *et al.*, 2001; Baruselli *et al.*, 2004). The addition of a progesterin-releasing device increased pregnancy rates in anestrous *Bos taurus* cows (Lamb *et al.*, 2001); however, this treatment has not been widely used in South-America for FTAI of beef cows and heifers. Conception rates have been often lower than 50% in *Bos indicus* cattle (Saldarriaga *et al.*, 2005; Pincinato, 2012). The addition of eCG has shown an improvement in P/AI in *Bos indicus* cows in postpartum anestrous (Pincinato, 2012) and in *Bos taurus* primiparous cows (Small *et al.*, 2009); but no improvement in P/AI have been reported in *Bos taurus* cows in good BCS ( $5.7 \pm 0.71$  BCS in the 1 to 9 scale; Marquezzini *et al.*, 2013).

### Fixed-time AI treatments for dairy cattle

In dairy cattle, FTAI protocols currently used in South-America are based on estradiol and progesterin and/or based on GnRH. Estradiol and progesterin treatments for dairy cattle are similar to those previously described for beef cattle, with reported P/AI between 35 to 55% (Bó *et al.*, 2009).

Ovsynch protocols have been used extensively in recent years for FTAI in dairy cattle in USA (Caraviello *et al.*, 2006). The protocol currently used in dairy cattle consists of an injection of GnRH followed by PGF 7 days later, a second injection of GnRH 56 h after PGF treatment with fixed-time AI 16 h later (Brusveen *et al.*, 2008). The rationale for this protocol is that the first GnRH will induce LH release and ovulation of a dominant follicle and emergence of a new follicular wave within 2 days. The administration of PGF 7 days later will induce luteolysis, and the second GnRH will induce LH release synchronizing ovulation of the new dominant follicle (Pursley *et al.*, 1995).

Recent studies have shown that the first GnRH resulted in ovulation in 44 to 54% of dairy cows (Bello *et al.*, 2006; Colazo *et al.*, 2009), 56% of beef heifers (Martinez *et al.*, 1999), and 60% of beef cows (Small *et al.*, 2009), and the emergence of a new follicular wave was synchronized only when treatment caused ovulation (Martinez *et al.*, 1999). If the first GnRH does not synchronize follicular wave emergence, ovulation following the second GnRH may be poorly synchronized (Martinez *et al.*, 2002b). Prevention of the early ovulations by addition of a progesterin-releasing device to a 7-day GnRH-based protocol has improved pregnancy rates in heifers after fixed-time AI (Martinez *et al.*, 2002b). Another approach to increase the number of cows responding to a GnRH-based protocol is to pre-synchronize estrus with two PGF treatments 14 days apart, with the last PGF given 10 to 12 days before the first GnRH (Moreira *et al.*, 2001; Galvão *et al.*, 2007). This protocol has been named Pre-Synch Ovsynch and has been shown to improve pregnancy rates in GnRH-based FTAI protocols in dairy cows.

The Ovsynch protocol has not been successfully used to synchronize cows in postpartum anestrous. This protocol appears to induce ovulation in a high percentage of anestrous dairy cows, but some of these cows have a subsequent short luteal phase (Gumen *et al.*, 2003; McDougall, 2010) resulting in lower conception rates than in cycling cows (Moreira *et al.*, 2001). Thus, although Ovsynch may induce ovulation in non-cycling cows, there is still likely to be a reduction in conception rates in these cows. One alternative treatment for cows in post-partum anestrous is to combine a progesterone releasing device with the Ovsynch protocol. Although an initial experiment done with high producing cows in North-America showed a significant improvement in P/AI (55.2 vs. 34.7%) for cows treated or not treated with progesterone releasing



devices at the time of the first GnRH (Pursley *et al.*, 2001), another review (Stevenson *et al.*, 2006) has shown that the results are surprisingly variable, and are probably related to the high progesterone clearance rate of the North-American high producing dairy cows managed in intensive systems (Wiltbank *et al.*, 2006). In a recent study, the addition of two CIDR devices increased pregnancy rates in cows in anovulatory anestrus (Bisinotto *et al.*, 2013). Another approach to increase progesterone concentrations during Ovsynch in North-America includes the administration of GnRH 6 or 7 days prior Ovsynch. These treatments are called Double Ovsynch (Souza *et al.*, 2008) or G6G (Bello *et al.*, 2006) and have been reported to increase P/AI. Nevertheless, in pasture-based dairy cows, in which liver progesterone clearance is not that high, the insertion of a progesterone-releasing device had resulted in significant improvements in P/AI (Veneranda *et al.*, 2008; McDougall, 2010).

### Equine Chorionic Gonadotropin (eCG) in dairy cattle

Contrasting results have been reported about the use of 400 IU of eCG at the time of removal of a progestin device in dairy cattle. In three experiments performed with lactating dairy cows in Argentina, P/AI were significantly higher ( $P < 0.01$ ) in cows treated with progesterone-releasing devices, estradiol and eCG (145/298; 48.7%) than in those treated with the Ovsynch protocol plus progesterone-releasing devices (117/298; 39.3%; Veneranda *et al.*, 2006, 2008). Furthermore, the treatment of lactating dairy cows with eCG in Brazil has shown differences in pregnancy rates among cows with lower body condition score (BCS  $< 2.75$ ; Souza *et al.*, 2009). They were higher in those treated with eCG (38.0%; 30/79) than in those not treated with eCG (15.2%; 12/79;  $P < 0.05$ ). Conversely, pregnancy rates did not differ in cows with BCS  $> 2.75$  and treated (29.9%; 93/311) or not treated (33.1%; 100/302) with eCG (Souza *et al.*, 2009). However, in a more recent study, adding either 400 or 600 IU eCG to FTAI protocols was inefficient to alter follicular and luteal dynamics and increase P/AI in high producing dairy cows that were more than 150 days in milk in Brazil (Ferreira *et al.*, 2013).

An experiment was performed in New Zealand to compare the reproductive response of lactating, seasonally calving, dairy cows diagnosed by rectal palpation with anovulatory anestrus (Bryan *et al.*, 2010). In the first experiment, the addition of eCG to an estradiol and progestin treatment resulted in increased P/AI (eCG: 48.9%;  $n = 432$  vs. no eCG: 43.1%;  $n = 420$ ;  $P = 0.059$ ), especially in cows older than 5 years of age.

A follow-up study was designed to evaluate the effect of adding eCG to a GnRH-based synchronization programs in New Zealand (Bryan *et al.*, 2013). Cows of 15 commercial dairy farms ( $n = 1991$ ) were selected for inclusion on the basis of non-observed estrus by 7 days

prior to the planned start of mating (PSM) and diagnosed with anovulatory anestrus by rectal palpation at the beginning of the trial. Cows were included for treatment according to the trial protocol (2 x 2 factorial). On day 0, all cows received a Cue-Mate intravaginal device (Bioniche Animal Health, Australasia) and an injection of 100  $\mu\text{g}$  gonadorelin (GnRH; 1 ml Ovurelin, Bayer, New Zealand) and were randomly assigned to have their device removed and receive 500  $\mu\text{g}$  of cloprostenol (Ovuprost, Bayer) on day 6 or 7. Within each group cows were further subdivided to receive 400 IU of eCG (Pregnecol, Bioniche) or no eCG (controls) at device removal. All cows were AI to detected heats and if not detected in heat, cows received a second injection of GnRH between 52 and 70 h after Cue-Mate device removal and were FTAI 72 h after device pull. Primary outcomes considered were 7-day in calf rate (ICR); 28-day ICR; and days to conception (DTC). There were no significant differences between a 6- or 7-day program, and there were no 6/7-day program by eCG interactions. However, inclusion of eCG into either a 6 or 7 day GnRH and Cue-Mate synchronization program increased 7 day ICR ( $P < 0.046$ ), 28 day ICR ( $P < 0.008$ ), and decreased median days to conception ( $P < 0.005$ ). Overall, treatment with eCG increased 28 day ICR from 50.4 to 56.2%. These results confirm that the addition of eCG into synchronization protocols for anestrus cows in seasonally calving herds has a significant value to get more cows pregnant at the beginning of the breeding season.

Two follow-up experiments were conducted in Argentina, to evaluate the effect of adding eCG to GnRH plus progesterone protocol on follicular dynamics and pregnancy rates in lactating Holstein cows in a mixed management system (i.e. 35% pasture and 65% grain and silage). In experiment 1, 40 Holstein cows with  $65.0 \pm 3.6$  days in lactation, BCS of  $2.9 \pm 0.1$  (scale of 1 to 5), and producing  $32.3 \pm 3.1$  l of milk were used. On day 0, all cows received a Cue-Mate device and 0.05 mg leirelin (GnRH, Biosyn-OV, Biotay, Argentina). On day 6, cows were divided into two groups to receive 0.15 mg of D (+) cloprostenol (PGF, Bioprost-D, Biotay) at the time of removal of the Cue-Mate device (Group 6 days) or 24 h after (Group 7 days). Furthermore, each group was subdivided (2 x 2 factorial) to receive 400 IU eCG (Pregnecol, Bioniche) upon removal of the Cue-Mate or no other treatment at that time. All cows received a second GnRH 56 h after Cue-Mate removal and were FTAI at 72 h after Cue-Mate pull. The cows were examined by transrectal ultrasonography to determine ovulation rate to the first GnRH and to the second GnRH. On day 0, 32/40 of the cows had a CL. Ovulation to the first GnRH was 75% (30/40), with no differences between groups ( $P = 0.45$ ). There were no differences between cows treated or not with eCG on the characteristics of the ovulatory follicle ( $17.1 \pm 0.4$  mm vs.  $16.2 \pm 0.5$  mm;  $P > 0.14$ ) and plasma progesterone



concentrations in the ensuing luteal phase ( $7.6 \pm 0.5$  vs.  $7.1 \pm 0.5$  ng/ml progesterone,  $P > 0.6$ ). In experiment 2, 453 lactating cows were synchronized with the same treatments evaluated in experiment 1. P/AI were higher in cows treated with Cue-Mates for 7 days (86/227, 38%) than those treated for 6 days (61/226, 27%;  $P < 0.01$ ). However, there were no differences between the cows treated (75/227; 33%) or not treated (72/226, 32%;  $P > 0.8$ ) with eCG. Collectively, the results from these studies show that the addition of eCG does not increase pregnancy rates in Holstein cows treated with Ovsynch, suggesting that the addition of eCG would increase P/AI only in cows which are non-cycling and/or nutritionally restricted and with only small follicles that would fail to ovulate after the first and/or second GnRH. Higher producing dairy cows, which usually have large size follicles, may not be benefited by the extra stimulation of the growth rate of the dominant follicle induced by eCG.

### The 5-day Cosynch protocol

Bridges *et al.* (2008) compared a 7-day Cosynch protocol plus progestin device with FTAI at 60 h and a 5-day Cosynch protocol with FTAI at 72 h in postpartum beef cows. In that study, P/AI was 11% higher with the 5-day protocol. Santos *et al.* (2010) reported similar findings in dairy cattle. The hypothesis proposed was that the 5-day protocol provided for a longer proestrus with increasing estradiol concentrations due to continuous gonadotropin support for the dominant follicle. The ovulatory follicle of cows in the 5-day program benefited from this extra time and additional gonadotropin support. However, due to a shorter interval between the first GnRH and induction of luteolysis in the 5-day protocol, two injections of PGF 6 to 24 h apart were necessary to induce complete regression of the GnRH-induced CL.

More recently, Kasimanickam *et al.* (2012) reported that heifers inseminated at 56 h in a 5-day Cosynch protocol had, on average, a 10.3% higher P/AI than heifers inseminated at 72 h. In addition, Colazo *et al.* (2011) showed that P/AI did not differ between a 5-day and 7-day Cosynch protocols with a single administration of PGF in dairy heifers. In that study, the use of the first GnRH in the 5-day Cosynch protocol also did not seem to be necessary as P/AI did not differ when it was not used. Conversely, Lima *et al.* (2011) observed an increased P/AI in dairy heifers receiving the final GnRH concurrent with AI at 72 h after PGF compared to 16 h before AI. However, they also showed no benefit of a first GnRH. In a follow-up study, P/AI were greater in heifers receiving GnRH at CIDR insertion, but only when two PGF were administered at CIDR removal and 24 h later.

We recently carried out an experiment to evaluate a new treatment based on estradiol but with a prolonged proestrus (de la Mata and Bó, 2012). In the first study 28 *Bos taurus* beef heifers, that were 16 and

17 months of age were randomly divided into two groups. Heifers in Group 1 (EB 6-day,  $n = 14$ ) received 2 mg EB and an intravaginal device with 0.6 g of progesterone (Emefur 0.6 g, Merial Argentina SA) whereas those in Group 2 ( $n = 14$ ) were treated with the 5-day Cosynch. All heifers received 150  $\mu$ g of D-cloprostenol (Emefur, Merial) at device removal and were FTAI and received GnRH 72 h later. All heifers were examined by ultrasonography to monitor follicular development and ovulation. The initiation of a new follicular wave occurred earlier ( $P < 0.05$ ) in heifers treated with GnRH ( $2.1 \pm 1.0$  days) than in those treated with EB ( $3.7 \pm 0.9$  days). However, ovulation rate (91.6 vs. 92.8%), the diameter of the ovulatory follicle ( $11.7 \pm 0.2$  mm vs.  $12.0 \pm 0.5$  mm), the interval from PGF to ovulation ( $97.1 \pm 17.4$  h vs.  $95.1 \pm 12.5$  h) and P/AI (50.0 vs. 57.1%) did not differ ( $P > 0.5$ ) between heifers in the EB 6-d group and those in the 5-day Cosynch group. This treatment is still under investigation but in field trials done with 854 commercial beef heifers, overall P/AI with the EB 6-d treatment averaged 53.7% (range: 35 to 71.8%). In another follow-up study with Holstein heifers that is currently underway, preliminary P/AI were 59.2% (29/49) for the EB-6 d, 53.3% (24/45) for the 5-day Cosynch and 47.9% (23/48;  $P = 0.3$ ) for those in the control group, which were treated with the standard EB plus progestin protocol for 7 days. In conclusion, these treatments proved to be efficient for synchronizing ovulation in heifers. However, it is necessary to perform more studies with a large number of animals to determine if these treatments increase P/AI compared to the traditional EB plus progestin treatment currently used by most practitioners in South-America.

### Summary and conclusions

The use of protocols that control follicular development and ovulation has the advantage of being able to inseminate cows without the need for detecting estrus. These treatments have been shown to be practical and easy to perform by the farm staff, and more importantly, they do not depend on the accuracy in estrus detection. Treatments with GnRH and progestin-releasing devices and estradiol have provided for FTAI in beef and dairy cattle and the addition of eCG have been especially useful in increasing pregnancy rates in cows experiencing post-partum anestrus. Finally, shorter synchronization treatments that provide for a longer proestrus are an interesting new alternative for FTAI; however, more studies are needed to determine if these treatments increase P/AI compared to the traditional EB plus progestin treatment currently used by most practitioners in South-America.

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## Approaches to increase reproductive efficiency in artificially inseminated dairy cows

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### Abstract

Infertility has been linked to numerous factors in high producing dairy cattle in the last decades. The detection of estrus continues to present difficulties and, although progress has been made in regards to estrus synchronization and artificial insemination, the reproductive performance of dairy cows has not improved substantially. Moreover, in warm countries, summer heat stress is a major factor impairing fertility. This presentation expresses our views on factors of a non-infectious nature that affect the fertility of lactating dairy cows following AI. Special attention is paid to factors related to the cow and its environment and to some approaches to increase reproductive efficiency such as confirmation of estrus at insemination and the insemination procedure.

**Keywords:** bovine, cornual insemination, estrus, fertility, management, season.

### Introduction

In the past decades, infertility has been linked to numerous factors in high producing dairy cattle (Beam and Butler, 1999; Royal *et al.*, 2000). Reasons for the lower fertility have not been entirely linked to increased milk production (Lucy, 2001; López-Gatius, 2003; López-Gatius *et al.*, 2006; Garcia-Ispuerto *et al.*, 2007b). Therefore, a main objective in recent years has been to preserve fertility of dairy herds (Gosden and Nagano, 2002). New management practices (leading to the improved well-being of cows) can improve the health and fertility of dairy cows (Windig *et al.*, 2005), and there is a tendency towards a higher level of management in high producing compared to lower producing herds (Calus *et al.*, 2005). However, in spite of the progress of the knowledge in the reproductive physiology of the cow over the last several years, fertility has not substantially improved. Moreover, in warm countries, summer heat stress is a major factor impairing fertility. This presentation expresses our views on factors affecting fertility in high producing dairy herds. Special attention is paid to factors related to the cow and its environment and to some approaches to increase reproductive efficiency such as confirmation of estrus at insemination and the insemination procedure.

### Management factors

Infertility has been often associated with high milk production, but this problem is multi-factorial and cannot be solely attribute to milk yield (Lucy, 2001; López-Gatius, 2003). In three extensive studies including 24,366 AI we could not detect a negative effect of milk production on fertility (López-Gatius *et al.*, 2005a, b; Garcia-Ispuerto *et al.*, 2007b). However, extensive studies have made possible to link high milk production in individual cows to high fertility (Lucy, 2001; López-Gatius *et al.*, 2006). For example, early fertile cows (cows that become pregnant before 90 days postpartum) were those who produce more milk at day 50 postpartum (López-Gatius *et al.*, 2006).

The question is why higher producing cows are more likely to conceive at the beginning of lactation? Maybe because good management practices allow the expression of the genetic potential of these animals, whereas lower producer cows receive inadequate care. If genetic progress is linked to fertility declining, low producer cows should have a major chance for becoming pregnant earlier, and this is not the case. Probably, highly fertile and producer cows had the highest genetic merit within the herd and suffered a low negative energy balance during postpartum period (López-Gatius *et al.*, 2003). Conception rate and calving interval do not appear to be affected by the genetic merit of a herd (Mayne *et al.*, 2002), and the highest producing dairy cows in the herd are not necessarily those with the greatest negative energy balance or the lowest body condition score (Lucy, 2001; Grohn and Rajala-Schultz, 2000). Anyway, the findings of studies that have identified factors promoting fertility such as cows becoming pregnant during the first trimester postpartum should be incorporated in routine checks conducted on herds. Data derived from these types of study are often more interesting than those of studies examining factors related to fertility failure.

The effects of milking frequency on fertility have been extensively explored in dairy cattle (Barnes *et al.*, 1990; Erdman and Varner, 1995; Stelwagen and Knight, 1997). Some studies conclude that reproductive performance is unaltered by milking three times per day (Amos *et al.*, 1985; Barnes *et al.*, 1990; Kruip *et al.*, 2002), whereas others have related this milking frequency to reduced fertility (DePeteres *et al.*, 1985; Gisi *et al.*, 1986; Smith *et al.*, 2002). In a more recent

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study (García-Ispuerto *et al.*, 2007b) analyzing 10,965 inseminations, we demonstrated that a three times daily milking regimen reduced fertility by an odds ratio of 0.58, compared to milking two times per day. The strong negative effect of the three times daily milking routine observed in our study adds more fuel to the controversy over the effects of milk production on reproductive performance. Milking frequency was a risk factor for infertility, whereas milk production at AI not. This indicates that the decrease in fertility associated with milking frequency seems not to be directly related to the increased milk yield. It is likely that cows milked three times per day will be more influenced by the luteolytic effects of oxytocin release in response to udder massage at each milking (Silvia *et al.*, 1991). An equally plausible explanation is the additional stress induced by the extra milking event each day.

The bull and AI technician effects on fertility have been extensively reported. In several studies performed in our area we could determine that some bulls decreased fertility at an odds ratio range of 0.31 to 0.44 (López-Gatius *et al.*, 2005a; García-Ispuerto *et al.*, 2007b), whereas one single bull increased fertility by a factor of 4.7 (López-Gatius *et al.*, 2005b). According to DeJarnette *et al.* (2004), we should consider whether the decline on fertility of high producing dairy herds can be attributed sometimes to the male, as a logical question. The problem is that many environmental and herd management factors will affect fertility estimates of an inseminating bull (Foote, 2003). Therefore, a continuous control of seminal doses entering in a herd should be useful to promptly locate a negative bull. Concerning the AI technician, we could only assess this factor in one single study including 10,965 inseminations (García-Ispuerto *et al.*, 2007b). The different AI technicians caused great variation in pregnancy rates. The likelihood of pregnancy decreased by a factor of 0.25 when a cow was inseminated by the worst compared to the best inseminator. Two of 13 AI technicians performed bicornual insemination of all cows (half of the seminal dose, 4-5 cm deep into each uterine horn), whereas the remaining 11 AI technicians performed uterine body insemination. Better results were obtained from technicians performing deep AI, in agreement with previous reports (López-Gatius and Camón-Urgel, 1988; Senger *et al.*, 1988). This variation in pregnancy rates could be an important practical limitation for the success of AI and also for herd fertility. It is clear that some AI technicians inseminate cows less efficiently than others. Deep cornual AI requires more training of inseminators and, therefore, favors results (Senger, 1993; López-Gatius, 2000). However, irrespective of the use of deep AI, retraining and continuous control of inseminators should be considered.

### Environmental factors

Environmental factors such as the use of a bull in the herd; poor nutrition or the loss of the body

reserves (negative energy balance); and housing elements (concrete slatted or dirty floors) can affect fertility. However, most studies report the seasonal effect as a major environmental factor affecting fertility. Although heavy rain, strong wind or high humidity can reduce fertility, high temperatures have been strongly linked to low fertility (see review by López-Gatius, 2012).

The fertility indicators conception and pregnancy rate to first service have suffered a fall of 0.5-1% per year (Royal *et al.*, 2000; López-Gatius, 2003). However, when the results were stratified according to season, warm period resulted in an increased infertility, while cool period preserved fertility. Thus, average conception rates to first AI in 2000 were 43 and 22% for the cool and warm periods, respectively (López-Gatius, 2003). The continued increase in milk production has been the result of improvements of genetics, nutrition and management practices, which probably cope with the negative effect of milk production. During warm period, any stressor such as high temperatures, could compromise the benefits of milk production. Heat stress seems to induce the premature aging of oocytes (Edwards *et al.*, 2005; Schrock *et al.*, 2007; Andreu-Vázquez *et al.*, 2010). Metabolic demands due to high milk production added to stressful factors such as high temperatures can compromise the reproductive functions of cows (Labèrnia *et al.*, 1998; De Rensis and Scaramuzzi, 2003; López-Gatius, 2003).

In a study including the temperature humidity index (THI; García-Ispuerto *et al.*, 2007a), a negative effect of high maximum THI was found, especially 3 days before AI and at the day of AI. This index incorporates the effects of both ambient temperature and relative humidity (RH) in an index (Thom, 1958) and is widely used in hot areas worldwide to assess the impact of heat stress on dairy cows (Hahn, 1969; Fuquay, 1981). However, when temperature was analyzed alone, it was demonstrated that high temperatures on day 3 before insemination and 1 day after were correlated with low fertility. Thus, climate factors seem to be highly relevant for conception rate, especially during the period encompassing 3 days before to 1 day after AI. The use of the THI or temperature to control a farm environment would depend on the individual farm and on each environmental situation, but it is important to check temperature and humidity to know when to adopt cooling measures.

### Confirmation of estrus

Inseminating the cow is the final, but by no means the least important, step in the process of a good estrus detection practice in the herd. Despite the significant progresses in the development of estrus detection aids during the last decades, detection of estrus remains a major problem in the XXI century (Roelofs *et al.*, 2010). Incorrect estrus detection is the



most common and expensive cause of failure of AI programs. Cows are often falsely identified as being in estrus and inseminated when conception cannot occur (López-Gatius and Camón-Urgel, 1991; López-Gatius, 2000, 2011; Sturman *et al.*, 2000). Although professional inseminators palpate the reproductive tract of numerous cows every day, most are not trained to examine the uterus and ovaries and, therefore, to confirm estrus. This situation poses a serious practical limitation to the success of estrus detection procedures and AI. Estrous signs in pregnant cows make the situation even more difficult. Pregnant cows stood willingly to be mounted by another cow or bull at all stages of pregnancy (Thomas and Dobson, 1989) and the insemination of pregnant cows can cause embryonic mortality or abortion (Vandemark *et al.*, 1952). In fact, 19 (Sturman *et al.*, 2000) to 40% (Nebel *et al.*, 1987) of AI have been incorrectly performed in pregnant cows. Finally, the most accurate external sign of estrus, standing to be mounted, was only registered in 58% of estrous periods in a more recent study (Roelofs *et al.*, 2005). Thus, the first goal of any estrus confirmation program should be to positively identify estrus and to reject cows for insemination that are not ready for service or are pregnant. Through rectal examination of the bovine reproductive tract either by hand or by ultrasonography, an animal can be correctly diagnosed as being ready for service (Roelofs *et al.*, 2010).

### Feeling the ovaries

As ovulation approaches, the follicle feels very soft separating itself from the remainder of the ovary (Studer and Morrow, 1981; Keenan, 1984). Hereafter, the ovulatory follicle is rapidly evacuated during the process of ovulation, and this ovulatory depletion may be difficult to recognize, especially 12 h after ovulation (Hanzen *et al.*, 1999). Thus, the sequence of changes that the dominant ovulatory follicle goes through at palpation during the periovulatory period is: firm/soft follicle (young preovulatory follicle), followed by very soft follicle (mature preovulatory follicle), followed by evacuated follicle (follicle associated with ovulation). Since one of these three follicle types are usually present at the time of insemination, a recent study on 2,365 AI was designed to determine possible differences between the types of follicle firm/soft, very soft or evacuated in terms of their effects on fertility (López-Gatius, 2011). The likelihood of pregnancy decreased significantly by factors of 0.86 or 0.82 in cows with a firm/soft follicle inseminated during the cool or warm period, respectively, and by a factor of 0.09 in cows with evacuated follicles inseminated during the warm period, using as reference cows with a very soft follicle inseminated during the cool period (yielding the highest pregnancy rate). The state of the periovulatory follicle at insemination was clearly related to fertility and masked the effects of factors commonly affecting fertility such

as parity, days in milk at AI and inseminating bull. More importantly, these results suggest that by including ovarian follicle checks in artificial insemination routines, the success of this procedure could be improved.

### Site of semen deposition

During mating, the bull deposits several billion spermatozoa into the anterior vagina. However, since the cervix is a major obstacle for sperm transport, the number of spermatozoa that finally reach the uterine body usually does not exceed 1% of the ejaculated spermatozoa. In artificial insemination, semen is generally deposited directly into the uterine body, thus bypassing the cervix and permitting the use of a considerably reduced number of spermatozoa (López-Gatius, 2000). One of the most significant contributions to the successful commercial application of AI in dairy cattle breeding has been attributed to the highly trained inseminator (Foote, 1996). However, there has been a tendency to adopt routine insemination techniques and to ignore inseminator-related factors that, as it has been discussed above, can dramatically affect fertility (García-Ispuerto *et al.*, 2007b). Presently, the pregnancy rate after a single AI service is rarely higher than 40%, which is far from the 60% or higher rate commonly recorded in the 1960s (Olds, 1978). This drop in AI efficiency has prompted the suggestion of changing the site of semen deposition in cattle, based on the idea that deep uterine insemination should ensure the deposition of spermatozoa nearer to the uterotubal junction, which is thought to be the main sperm reservoir prior to ovulation (Hunter and Greve, 1998; López-Gatius, 2000). Therefore, several studies were designed to evaluate deep bicornual and unicornual uterine insemination in an attempt to approach inseminate to the uterotubal junction could favor the results (López-Gatius, 2012).

For the bicornual method, the inseminating catheter tip is guided into one uterine horn until resistance is met, and half of the semen dose deposited. In a similar manner, the remaining half dose is deposited in the opposite horn (Senger *et al.*, 1988). For deep unicornual insemination, ovaries are palpated per rectum to determine the side of ovulation, then the major curve of the uterine horn ipsilateral to the side of the preovulatory follicle is straightened out by gentle manipulation per rectum and the semen introduced into the cranial half of the horn (López-Gatius and Camón-Urgel, 1988). Significantly better results have been obtained after deep bicornual or unicornual insemination, although some authors question the efficiency of this technique (López-Gatius, 2012). Perhaps, the advantage of deep uterine insemination, either bicornual or unicornual, is that it allows the deposition of semen nearer to the uterotubal junction and reduces the chances of cervical deposition.



### Concluding remarks

From a scientific stand point, one should pay attention to cows becoming pregnant within 90 days as well as the state of the follicle at AI; whereas from a herd management stand point, one should pay attention to the training and re-training of the AI technicians, be careful not to inseminate pregnant animals and improve estrus detection. Semen providing bull should be frequently monitored; and THI and/or temperature measurements should allow us to decide to establish better cooling systems in the herds.

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## Semen evaluation techniques and their relationship with fertility

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### Abstract

This review summarizes those methods-established and emerging- of semen assessment whose outcome intends revealing its potential fertility and, as a carry-over concept, that of the sire whose semen we examined. The review does not, however, focus on the wide display of current techniques designed to explore specific or multiple sets of sperm attributes essential for fertilization but on two basic concerns present: the *heterogeneity* of the sperm suspension and the *multitude of attributes required* for each spermatozoon to be fertile; concepts that shadow our diagnostic capabilities. The review points out advancements in the exploration of the genome, the transcriptome, and the proteome of both spermatozoa and the seminal plasma which unveil how spermatozoa modulate their own survival and signal to the environment when displaying degenerative changes. Specific seminal plasma components, both among individuals and portions of the ejaculate, not only relate to survival but also signal differential immune tolerance by the female with a previously unattended linkage to fertility. Lastly it foresees how Cytomics, combining novel designed motility analyzers, flow cytometers and enhanced digital imaging shall dominate the landscape of andrological laboratories and enable quick determinations on huge sperm numbers for markers highly relevant to sperm function and hence, for fertility.

**Keywords:** comparative sperm evaluation, fertility estimation, *in vitro* methods, semen analysis, sperm quality.

### Introduction

Over the past decade, we have experienced an explosive development of *in vitro* assays to determine sperm intactness and measurement of sperm function that helped andrological diagnosis and the optimization of semen processing methods, as summarized in multiple reviews (Rodríguez-Martínez and Larsson, 1998; Graham, 2001; Katila, 2001; Rodríguez-Martínez, 2003, 2006, 2007b; Parkinson, 2004; Graham and Moce, 2005; Guilan *et al.*, 2005; Rijsselaere *et al.*, 2005; Petrunkina *et al.*, 2007; Rodríguez-Martínez and Barth, 2007; Moce and Graham, 2008). However, conventional semen evaluation is still often restricted to

determinations of sperm numbers, sperm motility and sometimes, but rather sparsely, sperm morphology. The main reason behind this restriction is the fundamental axiom that an ejaculate must contain above a certain number of motile, morphologically 'normal' spermatozoa to achieve minimum sperm numbers reaching the oviducts for eventual participation in the complex process of fertilization, finally leading to the safe development of the embryo(s) (Rodríguez-Martínez *et al.*, 2005; Rodríguez-Martínez, 2007a; Holt, 2011).

More and more methods are now available for semen evaluation that not only make it possible to disclose the level of 'normality' of the male genital organs but also the capability of spermatozoa (mostly related to their membranes but also their metabolomics) to interact with the surrounding fluids (seminal plasma [SP], female genital fluids, and *in vitro* culture media), cells (epithelia, cumulus cells, oocytes), or extracellular material (hyaluronan coating, the zona pellucida [ZP]) before fertilization. Methods are also available to disclose the status of the different organelles, the intactness of the nuclear genome and of the available transcriptome; all related to the capability to initiate early embryo development. Although some of these methods, particularly those of an '*omic*' nature, are yet restricted to the research bench, the accompanying development of relevant instruments, from Computer Assisted Sperm Analysers for motility or morphology (CASA respectively ASMA) to bench-model flow cytometers (FCs), are making assays accessible for clinical diagnostics and for semen processing for assisted reproduction. Yet, many of these methods are only of limited value for prediction of fertility (Rodríguez-Martínez, 2003). This review aims to critically review advances in the methodology to assess semen and the capacity different assays have to prognose fertility. Particular attention is paid to new methods to determine DNA and transcript intactness; also to those biomimetic *in vitro* assays that, by resembling events during sperm transport, storage, and interaction with the female genital tract and the oocyte, best provide clues for sperm selection and the role of sperm sub-populations in the ejaculate.

### Conventional semen assessment and fertility

The currently used spermogram of ejaculated spermatozoa focuses (besides the aspects of pH and volume of the ejaculate) solely on the number of

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spermatozoa (per unit of volume, i.e. concentration or as total per ejaculate) and its motility (including sometimes its kinematic patterns, if a CASA instrument is used). Sperm numbers are a blunt variable in relation to fertility, and only when below possible 'threshold numbers' do we see a proven relation between sperm numbers and fertility (Tardif *et al.*, 1999; Christensen *et al.*, 2011).

The subjectively measured sperm motility has been statistically related to fertility even for post-thawed semen in bulls (Rodríguez-Martínez, 2003), and in pigs (Cremades *et al.*, 2005). Studies in other species yield erratic results, with large variation between laboratories, owing to operator bias and differences in numbers of breeding/female numbers used to determine fertility (Rodríguez-Martínez, 2006). Kinematic analyses using CASA have shown variable correlations between particular motility patterns, such as linearity, and field fertility (Bailey *et al.*, 1994; Holt *et al.*, 1997; Zhang *et al.*, 1998; Hirai *et al.*, 2001; Januskauskas *et al.*, 2001; 2003; Broekhuijse *et al.*, 2012). Combining motility patterns with other parameters of sperm function in AI dairy sires allowed, however, for fertility estimation (Januskauskas *et al.*, 2001). Major constraints for conventional CASA instrumentation relate to the few spermatozoa analyzed/sample, the variability between users (Ehlers *et al.*, 2011) which, combined with the high cost of the instrumentation, jeopardize their wider use (Feitsma *et al.*, 2011). Alternative instrumentation is now available (Qualisperm™, Biophos, Switzerland) based on another principle than the classical digitalization of centroids over time. This novel technology is based on correlation analysis of single particles (spermatozoa) in confocal volume elements. Individual spermatozoa are projected on a pixel grid of a CMOS camera and the algorithm analyzes the number of fluctuations by correlation function instead of trajectories. This system benefits from a high throughput (usually 4 fields per minute), analyzing >2,000 spermatozoa/sample, and has been thoroughly tested for several species (Tejerina *et al.*, 2008, 2009; Johannisson *et al.*, 2009).

Most often, the proportion of morphologically normal spermatozoa in the ejaculate of a bull is related to its fertility post-AI (Phillips *et al.*, 2004; Al-Makhzoomi *et al.*, 2008; Nagy *et al.*, 2013) reflecting, together with sperm numbers and sperm motility, the degree of normality of spermatogenesis and sperm maturation within a cohort of sires. Morphological abnormalities are always present in any ejaculate, but differ in their impact on fertility. Some are specific defects that hamper fertilization while others, such as the pear-shaped sperm head deviation, impair proper embryo development (Rodríguez-Martínez and Barth, 2007), thus calling in AI stud sires for frequent (2-month interval) detailed assessments of sperm morphology using wet and stained smears. The reliability of such analyses requires large numbers of

spermatozoa accounted for per sample, i.e. 200 per wet smear and 500 for stained sperm heads. The latter allows for determinations of defects with clear relation to fertility for their uncompensable nature such as pyriform sperm head shape as an expression of a defective chromatin condensation during spermiogenesis (Al-Makhzoomi *et al.*, 2008). Software for ASMA have been developed since the 1980's, and have now reached an acceptable reliability for the analyses of sperm head dimensions, although they cannot yet determine sperm abnormalities of other nature (Auger, 2010); nor are there clear relationships with fertility (Peña *et al.*, 2005b; Saravia *et al.*, 2007b; Gravance *et al.*, 2009).

### **Sophisticated tests of specific sperm attributes and function, do they prognose fertility?**

At specialized laboratories, biomarkers of sperm intactness of function proven relevant for fertilization are studied (Silva and Gadella, 2006), mostly to explore *in vitro* how relevant the interactions between the spermatozoa and the female genital tract, the oocyte vestments and the process of fertilization in itself are, including the early development of the embryo. Finally, the different outcomes are related to fertility (Rodríguez-Martínez, 2007b).

Integrity and stability of the plasma membrane is paramount, and methods vary, from microscopy in wet smears, the use of the membrane impermeable dye eosin (eosin-nigrosin test), exposure to a hypo-osmotic saline solution (HOS-test) to use of single or multiple fluorophores (reviewed by Rodríguez-Martínez and Barth, 2007). Either method has indicated significant correlations to fertility and can either use microscopy or flow cytometry (FC) can be used for screening (Kavak *et al.*, 2003; Nagy *et al.*, 2004; Saravia *et al.*, 2007a, Martínez-Pastor *et al.*, 2010; Hossain *et al.*, 2011; Petrunkina and Harrison, 2011; Balao da Silva *et al.*, 2013). Fluorophores are most advantageously used combined, for instance to determine subtle changes in permeability using SNARF-1, YO-PRO-1 and Ethidium homodimer, the so-called triple stain (Peña *et al.*, 2005a, 2007), related to phospholipid scrambling (Merocyanine-540, YO-PRO-1 and Hoechst 33342) or phospholipid asymmetry (Annexin-V/PI; Januskauskas *et al.*, 2003; Hallap *et al.*, 2006b; Peña *et al.*, 2003, 2005a, 2007; Saravia *et al.*, 2007a), all related to capacitation in several species and with a good correlation with fertility (Hossain *et al.*, 2011). Sperm capacitation includes, moreover, an influx of Ca<sup>2+</sup> to the sperm perinuclear and neck regions and flagellum, the generation of controlled amounts of ROS, as well as the phosphorylation of protein residues (Gadella and Van Gestel, 2004; Harrison and Gadella, 2005; O'Flaherty *et al.*, 2006; Tulsiani *et al.*, 2007; Fabrega *et al.*, 2011), steps that can be measured *in vitro* and, eventually, associated with the fertility of the males. Mapping of



intracellular  $\text{Ca}^{2+}$  levels in spermatozoa and of  $\text{Ca}^{2+}$  displacement, for instance using the CTC-technique has helped discriminate fertility among bull sires (Thundathil *et al.*, 1999; Gil *et al.*, 2000). The accompanying hyperactivated motility has however, shown a low relationship with fertility (Zhang *et al.*, 1998; Januskauskas *et al.*, 2001; Rodríguez-Martínez *et al.*, 2008).

Correlations between mitochondria status and fertility are variable, mostly owing to the changes in mitochondria functionality over time (Martínez-Pastor *et al.*, 2004; Hallap *et al.*, 2005b; Peña *et al.*, 2009) and sperm handling (Macías García *et al.*, 2012). Besides energy, sperm mitochondria produce by-products of the metabolism of oxygen, including superoxide which converts into the damaging hydrogen peroxide, a Reactive Oxygen Species (ROS), which is mostly, but not completely, converted to oxygen and water by the enzymes catalase or superoxide dismutase (also known as antioxidants or scavengers). A certain level of ROS is essential for sperm function, including fertilizing capacity, but only when it is kept at optimal levels by the antioxidant capacity of the seminal plasma (Awda *et al.*, 2009; Mancini *et al.*, 2009; Am-in *et al.*, 2011), via antioxidant enzymes such as paraoxonase-1 (PON-1, Verit *et al.*, 2009) or the sperm-present PON-2 (Vicente-Carrillo *et al.*, 2013, Linköping University, Sweden; unpublished). However, when excessive numbers of leukocytes are present in the ejaculate, or the semen is subjected to oxidative stress (as during cooling in the absence of SP or other natural scavengers), increased ROS generation, either extrinsic (leukocytes) or intrinsic (sperm neck cytoplasm in immature or morphologically abnormal mitochondria), causes a deterioration in sperm motility (Guthrie *et al.*, 2008), sperm membrane integrity through peroxidation of its lipids (LPO) as well as DNA breakage and cross linking of the chromatin (Aitken and West, 1990; Koppers *et al.*, 2008) all leading to fertility deterioration. ROS levels are therefore very variable, making their proper determination difficult, albeit yet possible using the probe hydro-ethidine or through measurement lipid peroxidation (LPO) levels in the membrane lipid bilayer by using the 5-iodoacetamidofluorescein probe family (BODIPY- $\text{C}_{11}$ ®; Guthrie and Welch, 2007; Aitken *et al.*, 2007; Ortega-Ferrusola *et al.*, 2009a).

Acrosome intactness, a pre-requisite for fertilization, can be readily examined *in vitro* using phase contrast microscopy (Rodríguez-Martínez *et al.*, 1998) or be examined by fluorophore linked lectins by multi-parametric analysis (Nagy *et al.*, 2003, 2004). Yet, correlation between acrosome status and fertility are variable (Rodríguez-Martínez, 2007b).

Spermatozoa from human, boars, and bulls contain the hyaluronan (HA) receptor CD44 in their plasma membrane (Huszar *et al.*, 2003; Tienthai *et al.*, 2003; Bergqvist *et al.*, 2006, Vicente-Carrillo *et al.*,

2013, Linköping University, Sweden; unpublished) and should thus bind to solid state HA depots (PICSI, Sperm Selection device, USA, Huszar *et al.*, 2007), a technique to trap only mature spermatozoa that are able to react with the HA and depict some degree of hyperactivated-like motility pattern, ideal to select best spermatozoa for ICSI in human and was later used for stallion spermatozoa (Colleoni *et al.*, 2011), but numbers are low to determine a true relation to fertility.

The effective binding of the spermatozoon to the ZP is a critical step in the process of fertilization. The binding is species specific, only elicited by capacitated spermatozoa and it precedes acrosome reaction (AR) occurrence. Since ZP binding can easily be performed *in vitro*, several sperm ZP binding tests have been designed since the 1980's, either using whole ZP (oocytes), or hemi ZPs (cleaved oocytes; Rodríguez-Martínez, 2006). Although outcomes from ZP binding tests yielded significant correlations with AI-fertility in pigs (Lynham and Harrison, 1998; Ardon *et al.*, 2005) and bulls (Zhang *et al.*, 1998), the biological significance of the assay is questioned, mainly due to the fact that physiological sperm capacitation, and hence AR, do not involve all spermatozoa at a given time.

An alternative usually tested by many laboratories is the ability of presumably capacitated spermatozoa to penetrate into homologous oocytes *in vitro*, the so called oocyte penetration test, under conditions of *in vitro* oocyte maturation (Henault and Killian, 1995; Brahmkshtri *et al.*, 1999; Oh *et al.*, 2010) which seem to relate to fertility (Henault and Killian, 1995). However, since oocytes maturity level varies as well as not all spermatozoa at a given time are capacitated and prompted to engage in ZP penetration; there is variation in penetration rates which do not mirror possible fertility differences among sires.

Different end points in fertilization and subsequent early embryo development can be determined using *in vitro* fertilization (IVF); spermatozoa of various species have been repeatedly examined looking for a relationship between *in vitro* outcome(s) and field fertility when the same semen (or males) was used for AI. In most cases, the approaches were retrospective, i.e. the fertility levels of the semen or males used were already known and only a few were really made prospective, i.e. the semen was coded, used *in vitro* and the outcomes used to calculate an 'in vitro fertility' that was thereafter contrasted to the 'real' fertility in the field. It was soon apparent that significant relations appeared when the semen used had wide variations in fertility, and results could be accepted as reliable when the conditions for IVF were of a certain stringency and stability, i.e. low sperm numbers used, same levels of success in a control line over time, not major differences between cleavage and morula/blastocyst yields. Unfortunately, most studies (Rodríguez-Martínez, 2007b) had only low to medium relationships with fertility, being lowest for



morula/blastocyst rates.

Di-thio-treitol (DTT) and detergents (such as sodium dodecylsulphate) have been used to study the relative capacity of sperm nuclei to decondense *in vitro*, attempting to establish a method that resembles the process needed to form a male pronucleus during fertilization. The degree of decondensation can be assessed microscopically (Rodríguez *et al.*, 1985) or via Flow cytometry (FC) after Propidium-iodide (PI)-loading (Cordova-Izquierdo *et al.*, 2006) and has been related to fertility in sheep and pigs, respectively. Apoptotic-like changes and the presence of apoptotic markers have been detected in species where retained cytoplasmic droplets are common, such as the equine (Ortega Ferrusola *et al.*, 2009a, b, 2010). Although clearly related to storage and cooling, it remains unclear whether the presence of caspases is biologically relevant for male fertility.

Methods for *in vitro* separation of spermatozoa for robustness have been described (Rodríguez-Martínez *et al.*, 1997) with a major focus on the fact that spermatozoa in a normal semen sample usually show a typical progressive, innate linear motility; linearity that is used to surpass natural barriers such as the cervix, where they migrate along sialic acid rich mucus filled deep furrows. Assays exploiting the fact that spermatozoa have an innate tendency to migrate into most media (often culture medium but also more complex preparations of varying viscosity) brought in contact with a semen sample (swim-over, swim-down, swim-up) have been used to mimic *in vivo* events. This simple procedure has proven to select for sperm motility and membrane integrity, essential parameters for fertilization (Rodríguez-Martínez *et al.*, 1997) and has proven valuable for fertility prognosis, since the number of viable spermatozoa post swim-up reflected the innate fertilizing capacity of the tested semen sample (Zhang *et al.*, 1998; Hallap *et al.*, 2005a, b, 2006a). Viscosity, often associated with additives of the swim-up media has improved the results, basically by mimicking the *in vivo* situation (Rodríguez-Martínez, 2007b; Hunter *et al.*, 2011). Hyaluronan, a component of the oviductal fluid and the cumulus cell cloud (Rodríguez-Martínez *et al.*, 2001) has proven an excellent additive since it increased viscosity to the right proportion *in vivo* and selected for fertilizing capacity (Shamsuddin and Rodríguez-Martínez, 1994). As a follow-up, artificial (hyaluronate-based, not sialic-based) cervical mucus has also been tested, albeit with less discriminative results (Al Naib *et al.*, 2011).

Novel methods have recently been developed using alternative multiple micro fluidic flow streams for sperm self-migration which allow for the sorting of motile spermatozoa in a similar fashion as *in vivo* (Wang *et al.*, 2011), although not suitable for the isolation of large sperm numbers, these latter methods appear promising when adapted for IVF, where low, quasi physiological sperm numbers are co-incubated

with oocytes (Suh *et al.*, 2006). Other methods have been put forward as substitutes for farm animals, where a higher output of an intact population is selected (Rodríguez-Martínez *et al.*, 1997; Samardzija *et al.*, 2006). Examples of these methods are the centrifugation through columns of adherent particles, Sephadex or glass wool, (Januskauskas *et al.*, 2005) or through discontinuous density gradients of silate coated silica spheres (Rodríguez-Martínez *et al.*, 1997). Centrifugation through a single column of species specific formulations of colloid (based on silate-coated spheres, the SLC method) has proven successful to harvest the most robust spermatozoa from any (raw or serially processed) semen suspension, in most species tested so far (Morrell and Rodríguez-Martínez, 2009, 2010; Morrell *et al.*, 2010). The selective power, which is clearly related to species differences in osmolarity and density of the colloid (Morrell *et al.*, 2011), is equally present in different volumes and sperm preparations, but -once again-, the selection is simply a mirror of the proportion of robust spermatozoa in a semen sample, and thus with low relationship to fertility.

### Sperm 'omics

The exponential advances in analytical molecular biochemistry, also named the 'omics revolution, have even involved sperm assessment. The 'omics revolution refers to the study of genes (genomics), and the function of their products (functional genomics) either as RNA transcripts (transcriptomics), proteins (proteomics) and the various metabolites (Aitken, 2010), opening our possibilities to determine how their presence or changes relate to cell function including fertility. Such endeavor is being made possible by the application of DNA sequencing, DNA microarrays, mass spectrometry, and protein arrays which, when proper interfaces and bioinformatic tools are available, may provide cues for sperm function (Carrell, 2008).

### Sperm genomics

Spermatozoa provide during fertilization a haploid genome with intact coding regions and regulatory regions for essential genes, copies that must be intact (i.e. should not contain single or double stranded DNA breaks). Mammalian spermatozoa have the most tightly compacted eukaryotic DNA, built up upon transformations during spermiogenesis where the sperm chromatin replaces histones first by transient proteins and then by protamines (Oliva and Castillo, 2011). Sperm chromatin can show different abnormalities related to compaction; from damage to the actual DNA physical integrity as single or double stranded DNA strand breaks, nuclear protein defects interfering with histone or protamine conversion and



DNA compaction; to chromatin structural abnormalities such as defective tertiary chromatin configuration. While the last named can imply defects in the decondensation of the nucleus before building the male pronucleus and impair fertilization, the other two can jeopardize embryonic development since the oocyte (albeit being able to repair a limited amount of sperm DNA damage) would not be able to correct those damages (Johnson *et al.*, 2011). Sperm DNA disorders also include mutations, epigenetic modifications, base oxidation and DNA fragmentation, the latter also related to sperm handling. Pertaining to its relevance, evaluation of the degree of DNA integrity has increased over the years (Barratt *et al.*, 2010). DNA fragmentation, by being considerably present in subfertile males, is considered the most frequent cause of paternal DNA anomaly transmitted to progeny. Damaged sperm DNA may be incorporated into the genome of the embryo, and participate or lead to errors in DNA replication, transcription or translation during embryo development, ultimately contributing to diseases in future generations (Katari *et al.*, 2009). Moreover, DNA damage may remain in the germ line for generations, a matter of concern related to the increasing use of ICSI (today even used in horses or pets; Aitken *et al.*, 2009). Sperm DNA fragmentation can be studied with many techniques, including staining with the DNA fluorophore PI which, in species where DNA compaction is not high, can present two types of staining, a dimmer (related to low sperm quality) and a brighter version (normal spermatozoa, Muratori *et al.*, 2008). Other classical methods to determine DNA damage are: (a) the single-cell gel electrophoresis assay (COMET), (b) the terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labelling (TUNEL), (c) the acridine orange test (AOT), (d) the tritium-labelled 3H-actinomycin D (3H-AMD) incorporation assay, (e) the in situ nick translation (ISTN), (f) the DNA breakage detection fluorescence in-situ hybridizations (DBD-FISH), (g) the sperm chromatin dispersion test (SCD, Halo) or the evaluation of (h) the degree of induced denaturation of the DNA (the so called Sperm Chromatin Structure Assay, SCSA) (Fraser, 2004; Evenson and Wixon, 2006; Tamburrino *et al.*, 2012). Most of the above methods can use fluorescence microscopy while SCSA and TUNEL are usually explored via FC. Although SCSA has been extensively used, the outcome provided conflicting relations to fertility in selected bull and boar sires (Rodríguez-Martínez and Barth, 2007; Christensen *et al.*, 2011; D'Occhio *et al.*, 2013) or unselected stallions (Morrell *et al.*, 2008). SCSA does not specifically identify the amount of DNA damage but rather its susceptibility to harsh treatment, whereas TUNEL does. A TUNEL/PI procedure is now available combining the accuracy of TUNEL and the differentiation of two sperm populations depending on PI intensity, of which one is probably participating in fertilization since the

observed damage has no relation to motility or morphology (Muratori *et al.*, 2008). Alternatively, use of dithiotreitol (DTT) to decondense sperm nuclei and inclusion of a stain for dead cells provides a higher accessibility to the TDNT enzyme of the TUNEL, alongside with the detection of DNA fragmentation in live spermatozoa (Mitchell *et al.*, 2011). Considering the above, TUNEL appears to be a more sensitive method to predict infertility than SCSA, as determined in a recent meta-analysis (Zini *et al.*, 2008).

### Sperm epigenetics

Noteworthy, not only DNA quality but also the packaging of the paternal genome (epigenome) is essential to embryonic development and fertility (Miller *et al.*, 2010; Jenkins and Carrell, 2011). Alongside genetic material, the spermatozoon also contributes with epigenetic components (i.e. other than DNA-coding changes that can alter or regulate gene expression) that affect early embryo development (Hales *et al.*, 2011). Processes such as DNA methylation, selective histone retention, sperm specific histones with tail modifications, other chromatin associated proteins, perinuclear theca proteins, organization of the DNA loop domain by the sperm nuclear matrix and of sperm born RNAs are included (Pacheco *et al.*, 2011; Yamauchi *et al.*, 2011). Microarray- and serial- analyses of gene expression assays of spermatozoa from several species have shown differential presence of regulatory non-coding RNAs (either long [lncRNAs] or short [microRNAs, small interfering iRNAs and Piwi-associated piRNAs; Ponting *et al.*, 2009] which provide the zygote with a unique set of paternal mRNAs (Krawetz *et al.*, 2011). These provide variable array signals, which correspond to the inherent variability among spermatozoa within an ejaculate, between ejaculates and individuals. Despite this, use of suppressive subtraction hybridization (Lalancette *et al.*, 2008), or of global RNA profiles of spermatozoa from fertile and infertile men (García-Herrero *et al.*, 2010), or bulls (using a cDNA collection on DNA microarrays) with different NRRs, could lead to the identification of transcripts (protein kinase and ADAM5P) associated with high sperm motility (Bissonnette *et al.*, 2009). More recently, semen from high- respectively low-fertility bulls provided, when examined with Affymetrix bovine gene chips, significant differences of specific transcripts associated with fertility (Feugang *et al.*, 2010). It is foreseen that microarrays shall be a determinant for future diagnostics.

### Sperm proteomics

The study of protein products expressed by the genome has dramatically expanded over the past decade, owing to multidisciplinary methodological and instrumental developments, but also due to the central



role of protein interactions in cell function (Cox and Mann, 2007; Brewis and Gadella, 2010; Baker, 2011; du Plessis *et al.*, 2011). Spermatozoa are, by being so highly differentiated, advantageous cells to study proteomics of specific compartments such as the membrane, which basically is the area of major importance for their role in interacting with their surroundings and the oocyte (Arnold and Frohlich, 2011). Despite this methodological development, proteomic studies of spermatozoa are still limited (Oliva *et al.*, 2009), yet leading to comprehensive sperm protein databases (Duncan and Thompson, 2007; de Mateo *et al.*, 2011) with numbers of proteins and fragments exponentially increasing over time towards several thousands. The proteins identified thus far cover the expected spectrum of function (from energy production to cell recognition), but few are accurately linked to (in)fertility, most of them being enzymes (Novak *et al.*, 2010a, b).

### **The seminal plasma, a forgotten cue for fertility prognosis?**

The main proteins of the SP belong to one of three groups: proteins carrying fibronectin type II (Fn-2) modules (as present in boar, stallion, bull or buck), spermadhesins (boar) or cysteine rich secretory proteins (CRISPs, stallion) and their bulk is, in most species, of vesicular gland origin (Kelly *et al.*, 2006). SP proteins, acting as adsorbed proteins to the plasma membrane, modulate several essential steps preceding fertilization, regulating capacitation, the establishment of the oviductal sperm reservoir, the modulation of the uterine immune response, and sperm transport through the female genital tract, as well as in gamete interaction and fusion. Therefore, SP proteomes have been assessed in relation to reproductive outcomes (either fertility levels or (in) fertility (Drabovich *et al.*, 2011; Milardi *et al.*, 2012), in several species. SP proteins have been identified as associated with high/low fertility in bulls (Killian *et al.*, 1993), isolated as Osteopontin (OPN) and Lipocalin-Type Prostaglandin D synthase (Gerena *et al.*, 1998; Cancel *et al.*, 1999). The latter has been always observed in the sperm-rich spurts of ejaculates in species with fractionated ejaculation, including the pig (Rodríguez-Martínez *et al.*, 2009, 2010, 2011). The OPN has been related to fertility in pig (IVF, Hao *et al.*, 2006, 2008) and stallion (Brandon *et al.*, 1999). Some SP proteins (SP-2, SP-3, SP-4, and clusterin) have been found in higher concentrations in stallions with low fertility scores (Novak *et al.*, 2010b). SP-1 is positively (Brandon *et al.*, 1999) or negatively (Novak *et al.*, 2010b) correlated with fertility and was suggested to be homologous to a bovine fertility associated protein described by Killian *et al.* (1993), probably OPN. Moreover, the abundance of CRISP3 in equine SP was positively correlated to 1st-cycle conception rate (Novak *et al.*, 2010b) suggesting the protein family

might have a role in fertility, as suggested for rodents and humans (Koppers *et al.*, 2011). The spermadhesin PSP-I, seems to be negatively related to pig fertility (Novak *et al.*, 2010a). SP cytokine levels vary among males. Variation in SP contents of TGF- $\beta$  lacks a linear relation to fertility (Loras *et al.*, 1999; O'Leary *et al.*, 2011). However, a female could express different levels of endogenous cytokines (relevant for embryo survival) depending on the exposure to SP from different males, which might thus relate to the often observed differences in embryo survival among sires (e.g. innate fertility; Robertson, 2007, 2010).

### **Have we reached full diagnostic and prognostic value?**

Assays and/or attributes tested differ in relation to fertility. For instance, membrane integrity evaluated via fluorometry (FC) appeared more closely related to semen fertility than sperm motility. Sample power is most relevant; assessing a hundred spermatozoa per sample or exploring thousands of them lead to unsecure relationship to fertility. Strength can be gained also by adjoining assays, even when this implies that some attributes are repeatedly measured. There is no risk in this, since spermatozoa that are tested with one assay are different from all others, so a battery of tests is always advantageous (Rodríguez-Martínez, 2003). Following that path, several groups have combined the results of *in vitro* tests of the same semen samples in analyses of multiple regression (Rodríguez-Martínez and Barth, 2007), yielding higher correlations with fertility even when being retrospective. Calculations of predicted fertility combining the outcomes of various methods of semen evaluation *in vitro* in multivariate analysis, before the fertility of the donor males was tested in the laboratory or the field, has proven valuable (Zhang *et al.*, 1999; Gil *et al.*, 2005; Ruiz-Sanchez *et al.*, 2006). This approach enabled identification of sub-fertile bulls, whose expected and real fertility was below the limit considered for sub-fertility (62% nonreturn rate), while the other young bulls predicted to have satisfactory fertility had nonreturn rates of  $\geq 65\%$ . Identification of sub-fertile sires had been obtained with other bull (Hallap *et al.*, 2004) and boar stud populations (Ruiz-Sanchez *et al.*, 2006). Interestingly, most sperm parameters (and to some extent even fertility) appeared maintained over the functional age of the sires, provided no pathologies are acquired between measurements (Zhang *et al.*, 1997, 1998; Hallap *et al.*, 2005b, 2006a). However, intrinsic variation between ejaculates within sire was always present, which requires analyses of many ejaculates.

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## Improving postcryopreservation survival capacity: an embryo-focused approach

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### Abstract

The major challenge for a greater dissemination of *in vitro* produced (IVP) bovine embryos is to improve embryonic survival after cryopreservation. The involvement of embryonic lipids on this issue is well documented. However, it has been recognized that not only the amount of lipids that affects embryo cryotolerance, but the embryo survival capacity after cryopreservation is a rather multifactorial event. In this review, some strategies to improve embryonic lipid composition and postcryopreservation survival by modifying the embryos themselves to make them more cryopreservable are overviewed. The use of semi-defined and defined serum-free culture media, the addition of some chemicals in the culture media to modify embryo lipid composition, and the modulation of embryo cell membrane fluidity by cholesterol or unsaturated fatty acids added to the culture media and oocyte/embryo donor nutritional management with a diet enriched in polyunsaturated fatty acids, were described as alternatives for the improvement of IVP embryo survival after cryopreservation.

**Keywords:** bovine, cryotolerance, cryosurvival, *in vitro* produced embryos, lipid.

### Introduction

The global *in vitro* production of bovine embryos has increased for the 6th consecutive year in 2011. The total number of *in vitro* produced (IVP) embryos transferred worldwide was 373,836. Brazil alone was responsible for 85% of the global market of IVP embryos (Stroud, 2012). This achievement can be attributed to the high number of oocytes recovered by ovum pick-up (OPU) of zebuine breed donors, mainly represented by Nellore (*Bos taurus indicus*) animals, allowing the commercial application of *in vitro* production on large-scale programs (Pontes *et al.*, 2011).

Embryo cryopreservation is an assisted reproductive technology that allows the storage of excess embryos derived from *in vitro* production and embryo transfer programs so they can be commercialized or transferred at the most convenient time. It is considered a strategy to overcome some logistic problems associated with the transfer of large numbers of fresh embryos and mainly for expanding the commercialization of embryos between countries (Sudano *et al.*, 2012c).

Despite the very good results associated with the fresh IVP embryo transfer, the use of cryopreserved embryos is extremely limited. The modest results of cryopreserved IVP embryos limit their application at the field conditions as it is successfully done with the semen in the artificial insemination. Even after many advances in embryo research over the past decades, embryo cryopreservation remains one of the most challenging biotechnologies of bovine reproduction, since the cryopreservation results are still inconsistent. This fact reflects directly in the lower number of cryopreserved embryos (Fig. 1) in Brazil (3 to 7%) and worldwide (7 to 8%) over the last years (Stroud, 2010, 2012; Viana *et al.*, 2010; Viana, 2012).

The most common approach to deal with the disappointing results of cryopreservation is to vary the cryopreservation procedures by altering, for example, the concentration and type of cryoprotectants, the time and temperature of the protocol, and the addition of additives (sugars or surfactants). Despite the fact that this approach usually results in improvements, they are often limited, what has led to increasing the efforts on an embryo-focused approach by modifying the embryos themselves to make them more cryopreservable (Seidel, 2006). Therefore, the objective of this review is to present some strategies for improving postcryopreservation survival capacity through an embryo-focused approach in order to produce an embryo more resistant to the cryopreservation.

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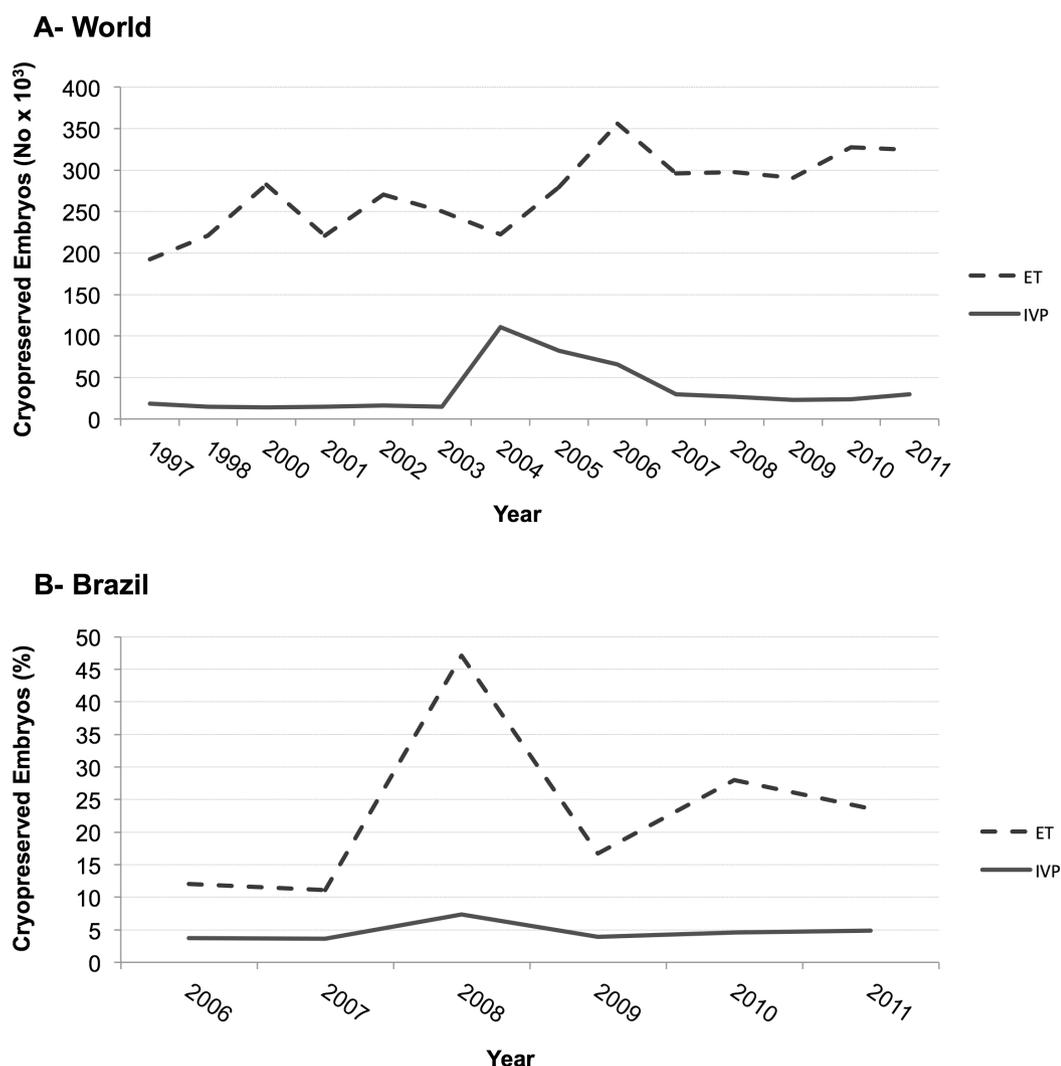


Figure 1. Total number and percentage of cryopreserved *in vivo* (ET) and *in vitro* (IVP) produced bovine embryos in the world (A) and Brazil (B). Data were obtained from IETS annual statistics and data retrieval committee report ([http://www.iets.org/comm\\_data.asp](http://www.iets.org/comm_data.asp)), and Brazilian record data (Viana and Camargo, 2007; Viana, 2009; Viana *et al.*, 2010; Viana, 2012).

#### Differences between *in vitro*- and *in vivo*-produced embryos and quality control of *in vitro* production systems

Since the first success of the cryopreservation of a mouse embryo (Whittingham *et al.*, 1972), several procedures were developed to cryopreserve embryos. These methods can be basically classified in two major strategies: slow freezing and vitrification. Despite that both of them are considered cryopreservation techniques, they have important conceptual differences. In the slow freezing system, extracellular water crystallizes resulting in increased osmotic gradient that draws water from the intracellular compartment until intracellular vitrification occurs. In the vitrification system, both intra and extracellular compartment vitrify after cellular dehydration has already occurred (Saragusty and Arav, 2011).

When comparing the results of these

cryopreservation methods based on the embryo origin, i.e. IVP *versus in vivo* produced (ET) embryos, ET embryos showed similar pregnancy rates (varying from 39 to 59%) for both slow freezing and vitrification procedures (Massip, 1987; van Wagtenonk-de Leeuw *et al.*, 1997; Inaba *et al.*, 2011), while the best results for IVP embryos (varying from 52 to 100% of re-expansion and from 36 to 93% of hatching/hatched rate) were achieved using vitrification (Nedambale *et al.*, 2004; Mucci *et al.*, 2006; Yu *et al.*, 2010; Inaba *et al.*, 2011). It is largely known that IVP embryos have a greater sensitivity to the cryopreservation techniques than ET ones (Leibo and Loskutoff, 1993) based on the fact that comparison of embryos from these two origins has demonstrated that the embryos do not survive equally in the different cryopreservation methods.

There are many morphological and metabolic differences between IVP and ET embryos, such as: very



electron-dense cytoplasm, loose blastomeres, buoyant density, metabolic abnormalities (“crabtree effect” and “unquite metabolism”), gene overexpression, apoptosis rate, lipid content, and postcryopreservation survival (Fair *et al.*, 2001; Abe *et al.*, 2002; Rizos *et al.*, 2002; Corcoran *et al.*, 2006; De La Torre-Sanchez *et al.*, 2006b; Mucci *et al.*, 2006; Leese *et al.*, 2008a; Côté *et al.*, 2011; Sudano *et al.*, 2011). All these alterations observed in IVP embryos can be attributed to the different *in vitro* culture conditions during oocyte maturation and embryo development that modulate the occurrence of these distinctive phenotypes (Lonergan *et al.*, 2003).

In the literature, the large amount of cytoplasmic lipid droplets observed in IVP embryos has been suggested to be the major cause of reduced postcryopreservation survival (Abe *et al.*, 2002; De La Torre-Sanchez *et al.*, 2006b; Mucci *et al.*, 2006; Barceló-Fimbres and Seidel, 2007a, b). Indeed, an increased amount of lipid droplets had a moderate correlation with the postcryopreservation survival. However, the embryo quality evaluated by the apoptosis rate had a strong correlation with the embryo survival after cryopreservation (Sudano *et al.*, 2012b), highlighting the importance of embryo quality after cryopreservation and suggesting that embryo cryosurvival capacity is a multifactorial event. Several factors are involved in the embryo cryotolerance, such as: lipid content, lipid composition, embryo metabolism, apoptosis, and global gene expression pattern (Sudano *et al.*, 2011, 2012a, b, c).

The goal during *in vitro* embryo production is try to mimick as much as possible the *in vivo* environmental condition to achieve a good quality embryo, which, in turn, could be cryopreserved more efficiently. Several researchers suggest a rigorous quality control during all steps of *in vitro* embryo production to obtain good results (Lane *et al.*, 2008; Hasler, 2010; Saragusty and Arav, 2011).

In this context, we explore in this review the important aspects that could affect IVP embryo quality and cryotolerance, namely: culture media composition (additives, salts, aminoacids, hormones, sugars, antioxidants, pH, and osmolarity), atmosphere (lower or higher oxygen tension), temperature, oocyte donor, semen, sire, and technician (Gardner, 2008; Leese *et al.*, 2008b; Feugang *et al.*, 2009; Hasler, 2010; Hugentobler *et al.*, 2010).

### Embryo lipids

There are evidences that at least four classes of lipids affect embryo survival after cryopreservation: triacylglycerides (TAG; mainly stored at the cytoplasmic lipid droplets), free fatty acids (FFA), cholesterol (Chol) and phospholipids (PL; cell membrane lipids).

The reason for the increased number of cytoplasmic lipid droplets in IVP embryos is unknown. However, it is speculated that it is related to fetal calf

serum (FCS) supplementation in the culture media. It seems that FCS increases embryo lipid content through: a) the lipoproteins from the serum are absorbed by the embryonic cells (Sata *et al.*, 1999); b) the embryo is induced to perform neosynthesis of triacylglycerides due to the presence of FCS (Razek *et al.*, 2000); and c) the FCS changes the function of  $\beta$ -oxidation in the mitochondria (Abe *et al.*, 2002). Another potential reason is that lipid accumulation occurs as an effect of abnormal energetic metabolism. An imbalance in the cellular oxidation-reduction process also occurs, affecting mitochondrial function and impairing metabolism of lipid complexes through  $\beta$ -oxidation (Abe *et al.*, 2002).

The lipid droplets present in the IVP embryos cytoplasm are mainly composed by TAG, the predominant lipid in the cytoplasm of mammalian cells (McKeegan and Sturmey, 2011). These stored lipids constitute an important source of energy for oocytes and embryos (Sturmey *et al.*, 2009). The estimated TAG content remained constant in a serum-free medium during embryo development (33 ng/embryo); however, in a serum-supplemented medium the TAG amount increased from 33 ng in 5-8 cell stage to 62 ng in hatched blastocysts (Ferguson and Leese, 1999). In addition, FCS also increased the total fatty acid amount compared with a serum-free media (74.2 vs. 57.2 ng, respectively), mostly represented by an increase in the palmitic (28.9 vs. 20.1 ng), stearic (18.0 vs. 13.1 ng), oleic (12.1 vs. 4 %), and palmitoleic (16.3 vs. 3.7 %), which are saturated (Reis *et al.*, 2003) and monounsaturated (Sata *et al.*, 1999) fatty acids.

On the other hand, PL are the most abundant lipid in eukaryotic cell membranes and their role in successful embryo cryopreservation remains poorly understood (van Meer *et al.*, 2008). Phospholipids, particularly phosphatidylcholines (PC) and sphingomyelins (SM), are structural units of functional membranes, and their composition determines the physicochemical properties of cell membranes, including fluidity, permeability, and thermal phase behavior (Edidin, 2003). We have recently reported that the PL profiles of bovine embryos vary between subspecies (*B. taurus indicus* vs. *B. taurus taurus*) and origin (IVP vs. ET) and that specific lipid species can potentially be used as biomarkers of embryonic postcryopreservation survival. These results indicate that not only the lipid amount but also the lipid composition accounts for embryo survival after cryopreservation (Sudano *et al.*, 2012c).

### Strategies to improve embryo lipid composition and postcryopreservation survival capacity

#### *Fetal calf serum-free media*

It is well known that FCS provides energy substrates, amino acids, vitamins, growth factors, and heavy-metal chelators. Although FCS has useful properties, its use has been associated with several



abnormalities, such as cell organelles modification, mitochondrial degeneration, gene expression modification, large offspring syndrome, increased lipid droplets number and reduced postcryopreservation survival (Abe *et al.*, 2002; Lazzari *et al.*, 2002; Rizos *et al.*, 2002, 2003; Sudano *et al.*, 2011). As a result, chemically-defined media (without FCS) have been developed (Keskintepe and Brackett, 1996).

It has already been described that it is possible to produce *in vitro* bovine embryos in defined (Block *et al.* 2010; Momozawa and Fukuda, 2011) or semidefined (Mucci *et al.*, 2006) serum-free media without affecting blastocyst yield and increasing embryo cryosurvival. In addition, a reduction of FCS concentration in the culture media alone was enough to decrease the lipid content and increase the postcryopreservation survival (Sudano *et al.*, 2011). The use of serum-free media has been considered as one of the first actions for the establishment of a successful *in vitro* embryo production system, allowing higher embryo survival after cryopreservation (Rizos *et al.*, 2003; Mucci *et al.*, 2006).

#### Use of chemical additives

Energetic substrate is promptly metabolized

through the glycolytic pathway (Fig. 2). However, the energetic metabolism during early embryo development (pre and post-compaction stage) is abnormal in IVP embryos (De La Torre-Sanchez *et al.*, 2006b). Under *in vitro* conditions, embryos show an increased activity of the glycolytic pathway and a consequently inhibition of oxidative phosphorylation pathway, characterized as “Crabtree effect” (Crabtree, 1929; Seshagiri and Bavister, 1991). A higher metabolic activity through the glycolysis impairs embryo development, because too little energetic substrate is partitioned to the pentose phosphate pathway (PPP) which is part of an important biosynthetic pathway (Wales and Hunter, 1990), by favoring lipid accumulation and rising cellular concentrations of lipid synthesis precursors (Rieger, 1992).

An interesting approach would be the use of phenazine ethosulfate (PES) in order to balance the energetic metabolism and reduce the lipid accumulation, by favoring the enzymatic reactions of PPP (Fig. 2), since this chemical oxidizes NADPH to NADP (De La Torre-Sanchez *et al.*, 2006a; Sudano *et al.*, 2011). The use of PES in the post-compaction period resulted in a reduction of the embryo lipid accumulation and an increase in the postcryopreservation survival (Barceló-Fimbres and Seidel, 2007b; Sudano *et al.*, 2011).

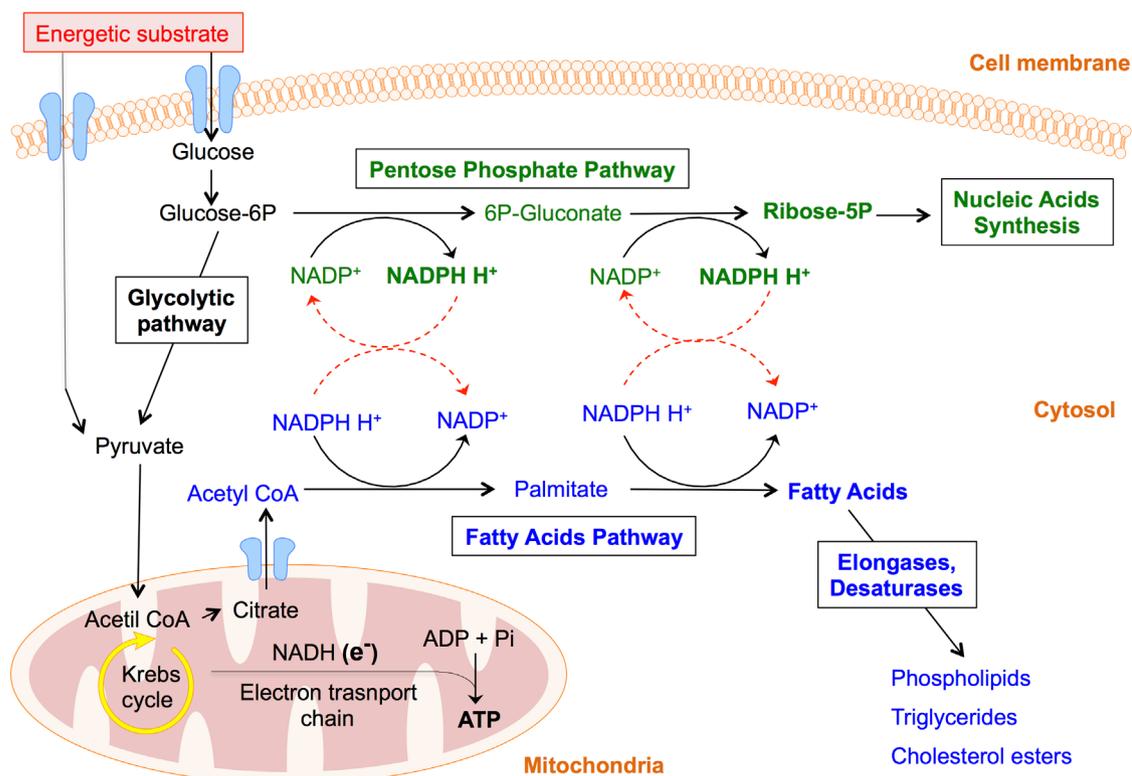


Figure 2. Energetic metabolism and phenazine ethosulfate (PES) mechanism of action in the preimplantation embryo. Energetic substrate is promptly metabolized in the glycolytic pathway (represented in black) through glycolysis within cytoplasm, followed by the krebs cycle and oxidative phosphorylation within mitochondria to produce energy in the form of ATP. PES reduces NADPH to NADP (dashed red arrows) favoring the pentose phosphate pathway (represented in green) with inhibition of the fatty acids pathway (represented in blue). Adapted from Barceló-Fimbres and Seidel (2007a).



Another chemical used to reduce lipid content of IVP embryos is the forskolin (Fig. 3A), a potent adenylate cyclase activator that stimulates the lipase activity through the cAMP/protein kinase pathway (Men *et al.*, 2006). Forskolin supplementation in the culture media reduced lipid content and increased the embryo survival after cryopreservation of bovine and porcine IVP embryos (Men *et al.*, 2006; Paschoal *et al.*, 2012).

More recently, L-carnitine, a small water-soluble molecule and cofactor of  $\beta$ -oxidation, was found to play an important role in the lipid metabolism (Sutton-McDowall *et al.*, 2012; Moawad *et al.*, 2013). This chemical is crucial for fatty acids (in form of acyl-CoA) translocation into the mitochondria (Fig. 3B), where they will be metabolized to acetyl-CoA through

$\beta$ -oxidation, and can be further metabolized in Krebs cycle and oxidative phosphorylation for ATP production (Sutton-McDowall *et al.*, 2012). L-carnitine also has an antioxidant activity protecting the cells from DNA damage (Abdelrazik *et al.*, 2009). Several beneficial effects of L-carnitine supplementation to the culture media have already been reported, including the improvement in the embryo development (Sutton-McDowall *et al.*, 2012), lipid metabolism and cryotolerance of bovine embryos (Takahashi *et al.*, 2012). The unique dual effects of L-carnitine enriching cellular lipid metabolism and providing antioxidative protection make it a chemical candidate for a non-invasive improvement of cryotolerance and developmental competence in IVP embryos (Takahashi *et al.*, 2012).

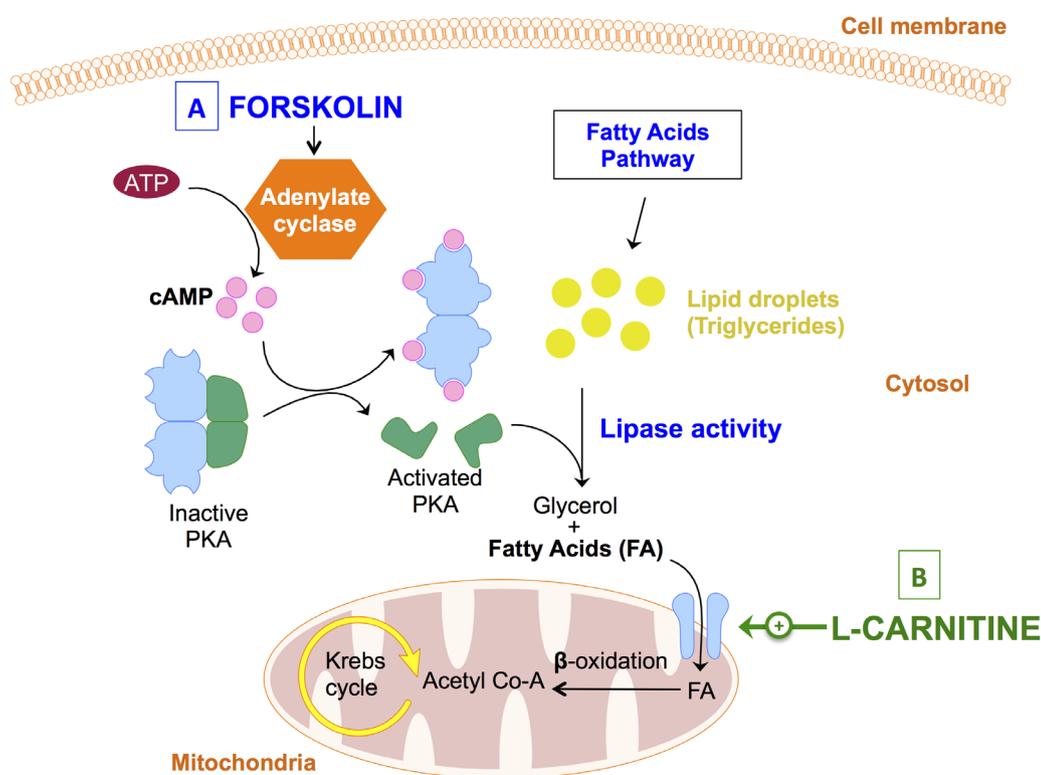


Figure 3. Mechanism of action of forskolin (A), a potent adenylate cyclase activator that stimulates the lipase activity through the cAMP / protein kinase (PKA) pathway, and L-carnitine (B), that favors fatty acid translocation into the mitochondria (in form of acyl-CoA) where they will be metabolized to acetyl-CoA through  $\beta$ -oxidation.

#### Cell membrane

In 1972, the structure of cell membrane was presented as the fluid mosaic model, which describes a cell membrane composed by a fluid bilayer of phospholipids oriented with the hydrophilic and hydrophobic portion to outside (extracellular and cytosolic compartments) and interior of the membrane, respectively (Singer and Nicolson, 1972).

The PL are composed by a three carbon backbone to which a phospho-head group is attached to

an end carbon of the backbone and two fatty acyl chains (fatty acids) are attached to the other carbons. Both the phospho-head groups and the fatty acids can vary in their composition and this will determine the properties of the cell membrane (Edidin, 2003). Indeed, it was reported that embryos with an increased abundance of unsaturated lipid species had greater cryosurvival (Sudano *et al.*, 2012c).

Cholesterol is another molecule present in cell membrane, and its level and the ratio between cholesterol and PL also affects the membrane fluidity (Horvath and



Seidel, 2006). Enriching the embryo cell membrane with unsaturated fatty acids and cholesterol to improve embryo cryotolerance has already been performed by two procedures, as follows: 1) membrane incorporation through its supplementation in the culture media; 2) nutritional management of oocyte/embryo donors and by offering a diet-rich with polyunsaturated fatty acids.

While the addition of cholesterol-loaded methyl- $\beta$ -cyclodextrin to the cryopreservation media had no effect on cryopreserved IVP bovine blastocysts (Pugh *et al.*, 1998), it seems to have a positive effect on vitrified oocytes as measured by an increase in the cleavage rate and number of eight-cell embryos (Horvath and Seidel, 2006), as well as an improvement in the nuclear maturation (Sprícigo *et al.*, 2012) after warming in comparison with the untreated group.

In addition, the unsaturated fatty acid supplementation (especially linoleic acid) in the culture media improved embryo cryotolerance (Hochi *et al.*, 1999; Pereira *et al.*, 2007) and reduced lipid content (Pereira *et al.*, 2007) of IVP embryos. Likewise, the oocyte/embryo donor nutritional management with a diet enriched in polyunsaturated fatty acids increased the cryosurvival of ewe oocytes (Zeron *et al.*, 2002) and porcine embryos (Kojima *et al.*, 1996).

### Conclusion

The major obstacle for a greater dissemination of the use of *in vitro* produced bovine embryos is their high sensitivity to the cryopreservation process. The involvement of the embryo lipids on this aspect is well documented. However, it has been recognized that not only the amount of cytoplasmic lipids affects embryo cryotolerance. The embryo survival capacity after cryopreservation is a multifactorial event. A rigorous quality control during all steps of *in vitro* embryo production is required to obtain a good quality and cryopreservable embryo. The use of a serum-free media, the addition of chemicals to change lipid metabolism, and the modulation of membrane lipid composition have been described as some alternatives for the improvement of the IVP embryo survival after cryopreservation

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## History and perspectives on bovine embryo transfer

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### Abstract

On a worldwide basis, more than 750,000 embryos are produced annually from superovulated donors and more than 450,000 embryos are produced using *in vitro* techniques. Superovulation and embryo collection are done as frequently as every 30 days. Cryopreservation and direct transfer of frozen-thawed embryos results in pregnancy rates near that of fresh embryos. Since the zona pellucida-intact *in vivo*-produced bovine embryo can be made specified pathogen-free by washing procedures, thousands of frozen embryos are marketed internationally on an annual basis. *In vitro* embryo production is used widely in countries like Brazil and Japan. Polymerase chain reaction (PCR) technology is currently being used for sexing embryos, and this technology is beginning to be used for “embryo diagnostics” and “embryo genomics”. Sex-sorted bovine semen is also readily available and is being used increasingly, especially for *in vitro* embryo production.

**Keywords:** export, genetic improvement, import, IVF, sexing.

### Introduction

For an historical perspective on assisted reproduction, the reader is referred to a comprehensive review of farm animal embryo transfer and its associated technologies (Betteridge, 2003). In brief, the first successful transfer of mammalian embryos was performed by Walter Heape in 1890. Heape transferred two four-cell Angora rabbit embryos into an inseminated Belgian doe, which subsequently gave birth to four Belgian and two Angora young (Betteridge, 2003). There were no reports of further success in mammalian embryo transfer until the 1920s, when several investigators again described embryo transfer in rabbits. Warwick and colleagues did considerable work on embryo transfer in sheep and goats in the 1930s and 1940s (Referenced in Betteridge, 1981, 2003), but it was Umbaugh (1949) who reported on the first successful embryo transfers in cattle. He produced four pregnancies from the transfer of cattle embryos, but all the pregnancies were terminated before full term. In 1951, the first embryo transfer calf was born in Wisconsin following the surgical transfer of an abattoir-derived day-5 embryo (Willett *et al.*, 1951; Referenced in Betteridge, 1981).

It was Rowson and colleagues who developed much of the technology that later found commercial use. Indeed, Betteridge (2003) has referred to Rowson as a founding father of embryo transfer in farm animals, and the International Embryo Transfer Society recognized his stature with the title of Founding Honorary President. In 1972, Rowson organized the first international course on bovine embryo transfer in Cambridge that brought together 13 veterinarians from around the world. Several of these registrants became the founding members of the International Embryo Transfer Society (IETS) and practitioners of commercial embryo transfer (Referenced in Betteridge, 2003).

The bovine embryo transfer industry as we know it today arose in North America in the early 1970's (Betteridge, 1981, 2003). Continental breeds of cattle imported into Canada were very valuable and relatively scarce because of international health and trade restrictions. Embryo transfer offered a means by which their numbers could be multiplied rapidly. However, it was private veterinary practitioners and small commercial companies who developed the technology for commercial use; they took techniques from the laboratory to the field. These pioneers encountered countless practical difficulties and founded the IETS to facilitate open discussion which they considered necessary if progress was to be made.

### Embryo transfer organizations

The IETS was founded in 1974, with 82 Charter Members, representing researchers, academics and veterinary practitioners from around the world (Carmichael, 1980; Schultz, 1980). The IETS became the main forum for scientific and regulatory exchange and discussion in the field of embryo transfer and associated technologies. The Proceedings of the Annual Meeting of the IETS, which were published as the first issue of *Theriogenology* each year, served as a yardstick with which to measure changes in emphasis and intensity of activity in embryo transfer. More recently, the IETS Proceedings have been published in the first issue of *Reproduction, Fertility and Development*. It is noteworthy, that since 1978, the proceedings of the Annual Meeting of the IETS have been published and available to registrants at the time of the meeting.

With the founding of regional embryo transfer organizations, a growing number of commercial embryo transfer practitioners have discontinued membership in

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the IETS in favor of their regional organizations. A growing number of the IETS members have been basic researchers representing government, industrial or academic institutions, including human medicine (Hasler, 2003). However, the IETS has played an important role in the dissemination of basic and applied information, allowing for the rapid growth of the embryo transfer industry. In particular, the Import/Export Committee of the IETS (now referred to as the Health and Safety Advisory Committee; HASAC) has been instrumental in gathering and disseminating scientific information on the potential for disease control with bovine embryo transfer. The Manual of the International Embryo Transfer Society "*A procedural guide and general information for the use of embryo transfer technology emphasizing sanitary procedures*" has become the reference source for sanitary procedures used in embryo export protocols (Stringfellow and Givens, 2010).

In 1982, the American Embryo Transfer Association (AETA) was formed to unite and organize the commercial embryo transfer industry in the USA, and in 1984, the Canadian Embryo Transfer Association (CETA) was formed. Objectives included the establishment of standards of performance and conduct, and a liaison with Federal agencies for both domestic and international embryo transfer. These associations also interact directly with breed associations, producer groups and international groups such as the IETS. They established standards of practice to provide confidence within each country, and internationally, for the utilization of embryo transfer technology. In this regard, their Certification Programs are integral in ensuring that Embryo Transfer Practitioners are technically and ethically competent in the handling of embryos used in international trade (Mapletoft and Hasler, 2005).

The Brazilian Embryo Technology Society (SBTE) was founded in Brasilia in 1985 (Rubin, 2005) as a private, not-for-profit organization of professionals dedicated to embryo transfer technology. Member's interests related primarily to cattle, horses and small ruminants, but also included swine, companion animals, laboratory species and wild and endangered species. Its stated goal is to serve as a forum for the exchange of information among practitioners, scientists, educators, livestock breeders and students as well as suppliers in the area of reproductive biotechnology. SBTE aimed to promote the science of animal embryo technology by encouraging effective research, disseminating scientific and educational information, maintaining high standards of ethics and cooperating with other organizations with similar objectives.

### **The application of commercial embryo transfer in cattle**

#### *Genetic improvement*

With the development of commercial embryo

transfer in the 1970s, its most common use in animal production programs was the proliferation of so-called desirable phenotypes. However, the University of Guelph introduced the concept of MOET (multiple ovulation and embryo transfer) in 1987 (Smith, 1988). They showed that MOET programs could result in increased selection intensity and reduced generation intervals, resulting in increased genetic gains. The establishment of nucleus herds and "Juvenile MOET" in heifer offspring was shown to result in genetic gains that approached twice those achieved with traditional progeny test schemes. It is noteworthy that prior to the Guelph work, most embryo transfer done in Canada was in beef cattle, whereas approximately 84% of the embryo transfer work done in Canada in 2011 involved dairy cattle. On the other hand, approximately 61% of embryo transfer work in the USA continues to involve beef cattle (Stroud, 2012).

Embryo transfer is now commonly used to produce AI sires from the top producing cows and proven bulls (Teepker and Keller, 1989). In addition, new genomic techniques are being used increasingly to select embryo donors; genomic analysis has become essential for the selection of bull dams to be used in embryo transfer (Seidel, 2010). Although economics would seem to preclude the use of embryo transfer techniques for anything but seed-stock production at this time, the commercial cattle industry has benefited from the use of commercial bulls produced through well designed MOET programs (Christensen, 1991). The success of MOET programs has also led to the use of this technology to genetically test AI sires (Lohuis, 1995); bulls were proven by production records from siblings rather than offspring (Smith and Ruane, 1987). It was possible to genetically test a bull in 3.5 years as opposed to 5.5 years using traditional progeny testing schemes, which also resulted in shortened generation intervals.

#### *Disease control*

Several large studies have now shown that *in vivo*-produced bovine embryos do not transmit infectious diseases. In fact, the IETS has categorized disease agents based on the risk of transmission by a bovine embryo (Stringfellow and Givens, 2000; Mapletoft and Hasler, 2005). Category 1 diseases include disease agents for which sufficient evidence has accrued to show that the risk of transmission is negligible, provided that embryos are properly handled between collection and transfer. This includes inspection of the zona pellucida at >50X magnification and washing/trypsin treatment procedures. Category 1 diseases include Enzootic bovine leukosis, Foot and mouth disease (cattle), Bluetongue (cattle), Brucella abortus (cattle), Infectious bovine rhinotracheitis, Pseudorabies in swine and Bovine spongiform encephalopathy. Category 2, 3, and 4 diseases are those



for which less research information has been generated. However, it is noteworthy that none of the infectious diseases studied have been transmitted by *in vivo*-produced bovine embryos. Consequently, it has been suggested that embryo transfer be used to salvage genetics in the face of a disease outbreak (Wrathall *et al.*, 2004).

#### *Embryo import-export*

The ability to utilize embryos in preventing the transmission of infectious disease makes them ideal for the international movement of animal germ plasm. The intercontinental transport of live animals also costs thousands of dollars, whereas an entire herd can be transported, in the form of frozen embryos, for less than the price of a single plane fare. Additional benefits of embryos for the international movement of animal genetics include reduced risk of disease transmission, reduced quarantine costs, a wider genetic base from which to select, the retention of the original genetics within the exporting country, and adaptation. Over the last 10 years, embryo import regulations for many countries have been simplified. In 2011, approximately 30,000 embryos were frozen in North America for export purposes, and 13,737 embryos were exported from Canada alone (Stroud, 2012).

Although handling procedures recommended by the IETS make it possible to safely export *in vivo*-derived embryos (Mapletoft and Hasler, 2005), it is a different story with embryos produced with *in vitro* techniques. The zona pellucida of *in vitro*-produced bovine embryos differs from that of *in vivo*-derived embryos (Stringfellow and Givens, 2000), and it has been shown that pathogens are more likely to remain associated with *in vitro*-produced embryos following washing than with *in vivo*-derived embryos. This has potentially serious ramifications for international movement, and protocols must be revised accordingly.

#### **Embryo transfer technology**

Although the applications and techniques associated with bovine embryo transfer have been reviewed extensively (Mapletoft, 1985, 1987), a brief historical perspective may be useful. Early investigators described non-surgical embryo recovery techniques (Rowson and Dowling, 1949), but these were not successful, and so all embryo recoveries and transfers were performed surgically in the early 1970s. These first commercial embryo transfer programs relied on mid-ventral surgical exposure of the uterus and ovaries with the donor under general anesthesia. This necessitated surgical facilities and limited the use of the technology in the dairy industry because the udder of dairy cows hindered mid-ventral access to the reproductive tract. It was not until 1976 that nonsurgical embryo recovery became sufficiently developed to be

used in practice (Drost *et al.*, 1976; Elsdon *et al.*, 1976; Rowe *et al.*, 1976). In the early 1980s, nonsurgical embryo transfer techniques (Rowe *et al.*, 1980) were also developed, allowing for on farm embryo transfer.

The embryo transfer industry grew rapidly in the late 1970s, both in terms of the number of practitioners and in the number of donors. Seidel (1981) reported that more than 17,000 pregnancies resulted from the transfer of bovine embryos in North America in 1979. More recently, Stroud (2012) reported that 572,432 *in vivo*-derived bovine embryos were transferred world-wide in 2011, of which 54% were transferred after freezing and thawing. In addition, 373,836 *in vitro*-produced bovine embryos were transferred, 85% of which were in Brazil. In 2011, North America continued to lead in commercial embryo transfer activity with collection of 54,837 donor cows and the transfer of more than 248,615 embryos (43% of all embryo transfers).

Although there has been no appreciable increase in the number of embryos produced per superovulated donor cow over the past 20 years, the importance of follicle wave dynamics (Adams, 1994) and methods for the synchronization of follicular wave emergence (Bó *et al.*, 1995, 2002), have simplified the means by which superovulation might be achieved, resulting in increased embryo production per unit time. Donor cows are being superstimulated more frequently than in the past (often every 30 days), and more embryos are being produced per year with no change in the actual superstimulation protocol. The application of similar procedures in recipients has made estrus detection, and the need to wait for animals to “come into heat” unnecessary, facilitating fixed-time embryo transfer (Bó *et al.*, 2002).

#### *Cryopreservation, direct transfer, and vitrification*

The development of effective methods of cryopreserving bovine embryos (Wilmut and Rowson, 1973; Leibo and Mazur, 1978) made embryo transfer a much more efficient technology, no longer depending on the immediate availability of suitable recipients. Pregnancy rates are only slightly less than those achieved with fresh embryos (Leibo and Mapletoft, 1998). Recently, the use of highly permeating cryoprotectants, such as ethylene glycol, has allowed the direct transfer of bovine embryos (Voelkel and Hu, 1992; Hasler *et al.*, 1997). In a study of the North American embryo transfer industry, pregnancy rates from direct-transfer embryos were comparable to those achieved with glycerol (Leibo and Mapletoft, 1998), and in 2011, more than 95% of frozen-thawed embryos were transferred by Direct Transfer (Stroud, 2012). In addition, a growing number of direct-transfer embryos are being transferred by technicians with experience in AI.

Freezing and thawing procedures are time-consuming and require the use of biological freezers.



Complicated embryo freezing procedures may soon be replaced by a relatively simple procedure called vitrification (Rall and Fehy, 1985). With vitrification, the embryo in high concentrations of cryoprotectants is placed directly into liquid nitrogen. As a result of the high concentration of cryoprotectants and the ultra-rapid rate of freezing, ice crystals do not form; instead the frozen solution forms a 'glass'. Since ice crystal formation is one of the most damaging processes in freezing, vitrification has much to offer in the cryopreservation of oocytes and *in vitro*-produced embryos. However, its greatest advantage is its simplicity. Vitrification is now widely used experimentally and *in vivo*-derived bovine embryos have been vitrified successfully in 0.25 ml straws for direct transfer (van Wagendonk-de Leeuw *et al.*, 1997).

#### *In vitro* embryo production

Bovine *in vitro* embryo production (IVP) is now a well-established and efficient procedure (Brackett and Zuelke, 1993). Moreover, ovum pick-up (OPU) at frequent intervals, in combination with *in vitro* fertilization, has improved and increased the yield of embryos from designated donors (Garcia and Salaheddine, 1998). *In vitro* fertilization has also been used to produce the thousands of embryos needed for scientific research, including efforts to produce embryonic stem cells; the constituent oocyte maturation and embryo culture techniques are integral parts of the procedures for cloning and transgenesis (Campbell *et al.*, 1996; Niemann and Kues, 2003). A few laboratories have also reported very modest successes in producing pregnancies with IVP embryos from calves (Duby *et al.*, 1996; Fry *et al.*, 1998; Taneja *et al.*, 2000), which offers the potential for decreasing generation intervals (Betteridge *et al.*, 1989). In addition, OPU has proven to be safe and very successful in pregnant cattle.

Several authors have directly addressed the question of using IVP as a substitute for *in vivo* embryo production (Sinclair *et al.*, 1995; Hasler, 1998; Bousquet *et al.*, 1999). At present, under commercial conditions in North America, IVP appears to be more expensive than conventional *in vivo* embryo production. For most breeders, this technology is an advantage only for extremely valuable cows which are infertile or fail to produce embryos after superstimulation. Indeed, the number of IVP embryos produced globally in 2011 as compared to 2010 was up less than 1%. However, IVP in Brazil in 2011 increased by 20% over 2010 resulting in 318,116 transferrable embryos. Brazil accounts for 86% of the world's total IVP. In 2011, 53,019 OPU sessions were performed in Brazil, yielding an average of 15 oocytes and 6 embryos per session. As a result, IVP numbers have surpassed that of *in vivo* embryo production in Brazil; it will be interesting to see if the trend continues for other countries in the world.

The efficiency of frozen IVP embryos will

likely determine the acceptance of IVP technology by other countries (Hasler *et al.*, 1995). So far, the majority of the IVP embryos have been transferred fresh, not frozen. However, data vary according to regions of the world. Worldwide 8% of the IVP embryos transferred in 2011 were frozen-thawed, while only 5% of IVP embryos were frozen in Brazil (Stroud, 2012). However, Brazil reports transferring more frozen-thawed IVP embryos each year, and results are improving.

#### Adoption of new technologies

Prenatal determination of sex potentially has great economic impact (Seidel, 2003) and the polymerase chain reaction (PCR) to determine the sex of bovine embryos is a service offered by many embryo transfer practitioners (Thibier and Nibart, 1995). However, embryo biopsy requires a high level of operator skill, and is an invasive technique resulting in disruption of the integrity of the *zona pellucida* and some reduction in the viability of the embryo, especially after cryopreservation. In the near future, PCR assays to identify other traits of economic importance will no doubt become available (Bishop *et al.*, 1995). Marker-assisted selection (MAS), based on identifying genetic markers for unknown alleles of valuable traits, probably has a similar future (Georges and Massey, 1991). Like genotyping of specific alleles, MAS can potentially be applied to embryo biopsies if sufficiently valuable markers can be identified. A PCR assay currently exists for simultaneous detection of the bovine leucocyte adhesion deficiency gene and the sex of embryo biopsies (Hasler, 2003). It is probable that PCR techniques will be developed that permit the analysis of a large number of markers from one biopsy leading to the concept of "embryo diagnostics". It is also likely that genomic testing of embryos with single-nucleotide polymorphism (SNP) technology will occur in the near future, again utilizing embryo biopsies and PCR technology (Seidel, 2010).

The flow cytometric technology used to separate X- and Y-bearing sperm into live fractions has been improved over the last 15 years (Johnson *et al.*, 1994; Johnson, 2000). Approximately 10 million live sperm of each sex can be sorted per hour (Seidel, 2003), with a resulting purity rate of >90%. In AI field trials, pregnancy rates following insemination with 1 million sexed, frozen sperm were reported to be 70 to 90% that of unsexed controls inseminated with 20 to 40 million sperm (Seidel *et al.*, 1999). A recent study which compared 574 calves produced from sex-sorted sperm with 385 control calves concluded that there were no differences in gestation, neonatal deaths, ease of calving, birth weight or survival rate to weaning (Tubman *et al.*, 2003). The disadvantages of flow cytometry are the slow speed of sorting, the decreased sperm viability (pregnancy rates), especially in superovulated donor cows, the cost of the semen, and the availability of



semen from specific bulls (Amann, 1999). It is likely that sexed semen will have the greatest use in IVP of bovine embryos in the near future.

### Summary and conclusions

Commercial embryo transfer in cattle has become a well established industry. Although a very small number of offspring are produced on an annual basis, its impact is large because of the quality of animals being produced. Embryo transfer is now being used for real genetic gain, especially in the dairy industry, and most semen used today comes from bulls that have been produced by embryo transfer. An even greater benefit of bovine embryo transfer may be that *in vivo*-derived embryos can be made specified pathogen-free by washing procedures, making this an ideal process for disease control programs or in the international movement of animal genetics. Techniques have improved over the past 40 years so that frozen-thawed embryos can be transferred to suitable recipients as easily and simply as artificial insemination is normally done. *In vitro* embryo production and embryo and semen sexing are also successful. A combination of embryo transfer using proven cows inseminated with semen from proven bulls, appears to be the most common use of bovine embryo transfer.

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## Innovative strategies for superovulation in cattle

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### Abstract

Although superstimulatory protocols in cattle are usually initiated during mid-cycle, the elective control follicular wave emergence and ovulation have had a great impact on the application of on-farm embryo transfer. However, the most commonly used approach for the synchronization of follicular wave emergence involves the use of estradiol which cannot be used in many parts of the world. Therefore, the need for alternative treatments has driven recent research. An approach that has shown promise is to initiate FSH treatments at the time of the emergence of the first follicular wave following GnRH-induced ovulation. Alternatively, it has been shown that it may be possible to ignore follicular wave status, and by extending the treatment protocol induce smaller follicles to grow and reach maturity and superovulate. Finally, the short half-life of pituitary FSH necessitates twice daily treatments which are time-consuming, stressful and subject to error. Recent treatment protocols have permitted superstimulation with a single or alternatively, two FSH treatments, reducing the need for animal handling during FSH treatments.

**Keywords:** eCG, estradiol, follicle wave, FSH, GnRH.

### Introduction

Although research efforts in recent years have resulted in little or no increase in the number of transferable embryos following superovulation, protocols that control emergence of the follicular wave (Bó *et al.*, 1995; 2002) and the timing of ovulation (Baruselli *et al.*, 2006; Bó *et al.*, 2006) have allowed the treatment of groups of donors, regardless of the stage of the estrous cycle and permitted fixed-time AI in donors, without the need to detect estrus. However, the most commonly used treatment for synchronization of follicular wave emergence for superovulation involves estradiol-17 $\beta$  or one of its esters, which cannot be used in many countries because of concerns about the effects of steroid hormones in the food chain (Lane *et al.*, 2008). The purpose of this paper is to review new developments in superovulation of beef cattle utilizing readily available pharmaceutical products.

Traditionally, gonadotropin treatments have been initiated during the mid-luteal phase,

approximately 8 to 12 days after estrus (reviewed in Bó *et al.*, 1995, 2002; Mapletoft *et al.*, 2002), around the time of emergence of the second follicular wave (Ginther *et al.*, 1989). However, a greater superovulatory response occurred when treatments were initiated on the day of follicular wave emergence, as apposed to 1 day before, or 1 or 2 days after wave emergence (Nasser *et al.*, 1993). Therefore, conventional treatment protocols have two drawbacks: 1) the requirement to have trained personnel dedicated to the detection of estrus, and 2) the necessity to have all donors in estrus at the same time in order to initiate treatments in groups of animals. We have recently summarized current superovulation protocols for cattle (Bó *et al.*, 2010; Mapletoft and Bó, 2012).

### Manipulation of the follicular wave for superstimulation

The ability to electively induce follicular wave emergence permits initiation of superstimulation without regard to the stage of the estrous cycle and eliminates the need for estrus detection or waiting 8 to 12 days to initiate gonadotropin treatments (Mapletoft *et al.*, 2009). In the 1990's, we reported on the use of progestins and estradiol to induce synchronous emergence of a new follicular wave (Bó *et al.*, 1995). This approach to superovulation in the cow has been reviewed extensively (Bó *et al.*, 2002; Mapletoft *et al.*, 2002; 2007). It has been used by practitioners around the world and has recently been incorporated into protocols that permit fixed-time AI of donors (Baruselli *et al.*, 2006; Bó *et al.*, 2006).

#### *Estradiol and progesterone*

The most common hormonal treatment to synchronize the emergence of a new follicular wave for superstimulation has involved the administration of 5 or 2.5 mg estradiol-17 $\beta$  or 2 mg estradiol benzoate, and 100 or 50 mg progesterone by intramuscular injection at the time of insertion of an intravaginal progestin device (reviewed in Bó *et al.*, 2002, 2006; Mapletoft *et al.*, 2002). The estradiol treatment causes a suppression of FSH release and follicle atresia. Once the estradiol is metabolized, FSH surges and a new follicular wave emerges, on average 4 days after treatment (Bó *et al.*, 1995). Superstimulatory FSH treatments are then

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initiated at that time, and continued for 4 days i.e., eight intramuscular injections. Prostaglandin (PGF) is normally administered with the fifth and sixth injections FSH and the progestin device is removed with the sixth injection. Estrus normally occurs approximately 48 h after the first PGF injection; inseminations are done 12 and 24 h after the onset of estrus.

#### *Fixed-time AI of donors*

Barros and Nogueira (2005) have developed what they refer to as the P-36 protocol in which the progestin device is left in place for up to 36 h after PGF administration (the reason the protocol is called P-36) and ovulation is induced by the administration of exogenous pLH (or GnRH) 12 h after withdrawal of the progestin device (i.e., 48 h after PGF administration). Since ovulation occurs between 24 and 36 h after pLH administration (Nogueira and Barros, 2003), fixed-time AI was scheduled 12 and 24 h after pLH, avoiding the necessity of estrus detection.

In a series of experiments in which the timings of ovulations were monitored ultrasonically, Bó *et al.* (2006) showed how synchronization of follicular wave emergence with estradiol could be incorporated into a protocol for fixed-time AI in donors without the need for estrus detection and without compromising results. Basically, FSH treatments were initiated on day 4 (day 0 was the time of estradiol and progestin treatment), PGF was administered on day 6 and progestin devices were removed in the morning of day 7 (preventing early ovulations and allowing late developing follicles to “catch-up”), followed by induction of ovulation with GnRH or pLH 24 h later. Fixed-time AI was done 12 and 24 h later. From a practical perspective, fixed-time AI of donors has been shown to be ideal for busy embryo transfer practitioners (Larkin *et al.*, 2006).

Studies in high-producing dairy cattle in Brazil have indicated that it is preferable to allow an additional 12 h before removing the progestin device (i.e., day 7 PM) followed by GnRH 24 h later (i.e., day 8 PM) with fixed-time AI 12 and 24 h later (Bó *et al.*, 2006). In *Bos indicus* breeds, it was found that it was preferable to remove the progestin device on day 7 PM, followed by GnRH 12 h later (i.e., day 8 AM; Baruselli *et al.*, 2006).

#### **Alternative approaches for follicle wave synchronization and superstimulation**

Recently, the use of estradiol has been restricted in countries such as USA, New Zealand and the European Union. This restriction created the need to develop treatments that do not involve the use of estradiol esters (Mapletoft *et al.*, 2009). However, the inaccessibility of effective synchronization tools leaves many embryo transfer practitioners with a serious dilemma.

#### *Follicle ablation*

An alternative to estradiol is to eliminate the suppressive effect of the dominant follicle by ultrasound-guided follicle ablation and initiate superstimulatory treatments 1 or 2 days later (Bungartz and Niemann, 1994; Bergfelt *et al.*, 1997). Initial studies involved the ablation of all follicles  $\geq 5$  mm (Bergfelt *et al.*, 1997), but we subsequently showed that it was only necessary to ablate the two largest follicles (Baracaldo *et al.*, 2000) to ensure that the dominant follicle was removed. Superstimulatory treatments may then be initiated 1 to 2 days later, at the time of emergence of a new follicular wave. Although this treatment has been shown to be highly effective (reviewed in Bó *et al.*, 2006), the disadvantage is that it requires ultrasound equipment and trained personnel which is only appropriate for embryo production centers, where all the donors are maintained at the same location; it is very difficult to apply in the field.

#### *GnRH or pLH*

GnRH has been reported to induce ovulation or luteinization of the largest follicle at the time of treatment (Macmillan and Thatcher, 1991), with emergence of a new follicular wave approximately 2 days later. However, it has also been shown that follicular wave emergence occurs only when treatment resulted in ovulation (Martinez *et al.*, 1999), and ovulation rates after GnRH treatment at random stages of the estrous cycle have been reported to range from 44.3% (Colazo *et al.*, 2009) to 85% (Pursley *et al.*, 1995). Therefore, the interval from GnRH treatment to wave emergence may not be as consistent as is required for superstimulation. Indeed, Deyo *et al.* (2001) reported unsatisfactory embryo production following synchronization of follicular wave emergence for superstimulation with GnRH or pLH. However, recent results from commercial embryo transfer practitioners (Hinshaw, 2009; AABP; personal communication; Steel and Hasler, 2009) and a research report involving 411 dairy donor cows (Wock *et al.*, 2008) have revealed much more promising results, with no difference in embryo production when compared to the use of an estradiol-based protocol. Basically, a progestin device is inserted at random stages of the estrous cycle and GnRH is administered 2 or 3 days later with superstimulation treatments beginning 1.5 to 2.5 days later. Although controlled and appropriately designed experimental studies must be designed to confirm these promising results, it is noteworthy that most of these protocols involve the insertion of a progestin device 2 or 3 days before GnRH is administered which may ensure the presence of an LH-responsive follicle.



### *Superstimulation at emergence of the first wave after GnRH-induced ovulation*

The first follicular wave emerges consistently on the day of ovulation (the day after the onset of estrus) in cattle (Ginther *et al.*, 1989). Nasser *et al.* (1993) showed that superstimulation can be initiated at the time of emergence of the first follicular wave, and first wave follicles have been shown to be as responsive to superstimulation as second wave follicles (Adams *et al.*, 1994). However, a progestin device must accompany gonadotropin treatments initiated at the time of emergence of the first follicular wave to ensure high oocyte/embryo quality (Nasser *et al.*, 2011).

An alternative approach for the synchronization of ovulation for superstimulation is to combine the use of GnRH and a progestin device as reported recently (reviewed by Bó *et al.*, 2008; Carballo Guerrero *et al.*, 2009). In this protocol, a persistent follicle was induced by administration of PGF at the time of insertion of a progestin device (Small *et al.*, 2009); then 7 days later, GnRH very effectively induced ovulation of the persistent follicle. The most user-friendly and efficacious protocol consisted of insertion of a progestin device and the administration of PGF on random days of the estrous cycle (day 0). Progestin devices are not removed and in fact stayed in place until the end of the superstimulation treatment protocol. GnRH or pLH was given on day 6.5 and FSH treatments were initiated 36 h later (i.e., day 8) at the expected time of ovulation (and wave emergence). By adding a second GnRH injection 24 h after progestin device removal (at expected onset of estrus), it was possible to do fixed-time AI with this protocol. Collectively, data suggest that superstimulation protocols involving the first follicular wave after a GnRH-synchronized ovulation can be used at a self-appointed time without estrus detection in groups of donors and with no decrease in embryo production.

### *Subordinate follicle breakthrough*

During a normal follicular wave, subordinate follicles regress because of decreasing concentrations of FSH, caused by the secretion of estradiol and inhibin by the cohort, and especially of the dominant follicle (Adams *et al.*, 1992, 1993). Small follicles require FSH to continue their growth, and evidence suggests that follicles as small as 1 mm in diameter will commence growth under the influence of FSH (reviewed by Adams *et al.*, 2008). We hypothesized that it would be possible for exogenous FSH to cause these follicles to grow to a diameter of 3 or 4 mm at which time the regular 4- or 5-day superstimulatory treatment protocol could be initiated. Assuming a growth rate of 1 to 2 mm per day, this should take 2 to 3 days. Thus, these follicles could be recruited by adding 2 to 3 days to the superstimulation treatment protocol. The presence of a

dominant follicle may not have any effect on superovulatory response under these circumstances because the exogenous FSH replaces that being depressed by the estradiol and inhibin. Indeed, Bó *et al.* (2008) successfully superstimulated donors at random stages of the estrous cycle, without regard to the presence of a dominant follicle, using this approach.

Alternatively, the 2 days of FSH pretreatment might be replaced with an injection on 500 IU of equine chorionic gonadotropin (eCG) 2 days before initiating FSH treatments. Indeed, Bó *et al.* (2008) have shown that pretreatment of poor responding donors with 400 IU of eCG 2 days before follicular wave emergence followed by FSH treatments 2 days later (Caccia *et al.*, 2000) resulted in an improved superovulatory response over that achieved previously without the use of eCG. Although not studied critically, it was hypothesized that the eCG recruited additional follicles into the wave.

More recently, we investigated the effect of lengthening the superstimulatory treatment protocol from the traditional 4 days to 7 days in order to recruit more follicles into the wave (García Guerra *et al.*, 2012). Lengthening the FSH treatment protocol to 7 days, without increasing the total amount of FSH administered, increased the percentage of follicles that ovulated, the number of ovulations and the synchrony of ovulations, and tended to increase the mean numbers of total ova/embryos, fertilized ova, and transferable embryos. In other words, the lengthened superstimulatory treatment protocol resulted in more follicles reaching an ovulatory size and acquiring the capacity to ovulate with an increased number of ovulations, and with no decrease in oocyte/embryo quality. It was concluded that prolonged FSH treatment protocols may be an effective strategy to recruit small follicles into the follicular cohort available for superstimulation, while providing the additional time needed for these follicles to reach an ovulatory size and acquire the capacity to ovulate. In addition, these results suggest that traditional 4-day superstimulatory treatment protocols may not provide adequate time for all follicles within the cohort to acquire the capacity to ovulate. This requires further study.

### **Reducing the number of FSH treatments in a superstimulation protocol**

Because the half-life of pituitary FSH has been shown to be 5 h in the cow (Laster, 1972), traditional superstimulatory treatment protocols consist of twice daily injections of pituitary FSH over 4 or 5 days (Mapletoft and Bó, 2012). This requires frequent attention by farm-personnel and increases the possibility of failures due to non-compliance. In addition, twice daily treatments may cause undue stress in donor cows with a subsequent decreased superovulatory response, and/or altered preovulatory LH surge (Stoebel and Moberg, 1982). Thus, simplified protocols may be



expected to reduce donor handling and improve response, particularly in less tractable animals.

More than 15 years ago, we reported that a single subcutaneous administration of FSH in beef cows in high body condition (>3 out of 5) resulted in a superovulatory response equivalent to the traditional twice daily treatment protocol over 4 days (Bó *et al.*, 1994). However, the results were not repeatable in Holstein cows, which had less adipose tissue (Hockley *et al.*, 1992). In a subsequent study in Holstein cows, the single injection was split into two, with 75% of the FSH dose administered subcutaneously on the first day of treatment and the remaining 25% administered 48 h later, when PGF is normally administered (Lovie *et al.*, 1994). Although superovulatory response was improved, it was still numerically less than the twice daily injection protocol.

An alternative to induce a consistent superovulatory response with a single injection of FSH would be to combine the pituitary extract with agents that cause the hormone to be released slowly over several days. These agents are commonly referred to as polymers, are biodegradable and non-reactive in the tissue, facilitating use in animals (Sutherland, 1991). We have recently completed a series of experiments in which FSH diluted in a 2% hyaluronan solution was administered as a single intramuscular injection, to avoid the effects of body condition. Overall, the single injection protocol resulted in a similar number of ova/embryos as the traditional twice-daily FSH protocol (Tribulo *et al.*, 2011). However, 2% hyaluronan was viscous and difficult to mix with FSH, especially in the field. We speculated that although more dilute preparations of hyaluronan were less efficacious as a single injection, their use could be improved by splitting them into two injections 48 h apart, as we had shown previously with subcutaneous injections of FSH. The split intramuscular treatment protocol consisted of diluting the FSH lyophilized powder with 10 ml of the reduced concentration hyaluronan solution and the administration of two-thirds of the total dosage of FSH on the first day, followed by a second injection with the remaining one-third of the total dosage of FSH 48 h later, when PGF is normally administered (Tribulo *et al.*, 2012). Overall, the numbers of transferable embryos did not differ among treatment groups (Control:  $4.0 \pm 0.8$ ; 1% hyaluronan:  $5.0 \pm 0.9$ ; 0.5% hyaluronan:  $6.1 \pm 1.3$ ). Data were interpreted to suggest that splitting the FSH dose in either reduced concentration of hyaluronan into two intramuscular injections 48 h apart would result in a superovulatory response comparable to the traditional twice-daily intramuscular injection protocol in beef cattle. Furthermore, the less concentrated solutions of hyaluronan were not difficult to mix with FSH, even under field conditions.

Barros *et al.* (2008) conducted an experiment in which Nelore cows were superstimulated with Folltropin-V over 3 days; the two FSH injections on day

4 were replaced by two injections of 200 IU of eCG. Donors in the control group were superstimulated with the conventional treatment of eight twice-daily decreasing doses of FSH over 4 days. Treatment with eCG significantly increased the number of ova/embryos and numerically increased the number of transferable embryos. Reano *et al.* (2009) examined the use of this protocol in Brangus cows and heifers and found that it resulted in an increased number of transferable embryos. It is tempting to speculate that a single injection of eCG might prove to be useful in a split-dose superstimulation scheme. Obviously, more research is required.

### Summary and conclusions

The use of protocols that control follicular development and ovulation has the advantage of being able to apply assisted reproductive technologies without the need for detecting estrus. These treatments have been shown to be practical and easy to perform by the farm staff. Estradiol is very efficacious in synchronizing follicle wave emergence for superovulation schemes, but is not available in many countries. Although the administration of GnRH to synchronize follicular wave emergence yields variable results, presynchronization with a progestin-releasing device has been shown to improve the response to GnRH allowing for superstimulation during the first follicular wave after ovulation, with results that did not differ from the use of estradiol. Lengthened superstimulation treatment protocols would appear to result in the recruitment of additional follicles into the wave and allow for the time needed for these follicles to acquire the capacity to ovulate. On the other hand, 4-day treatment protocols may not provide sufficient time for all superstimulated follicles to acquire the capacity to ovulate. Finally, the use of a hyaluronan-based, slow-release formulation has shown that it is possible to induce a consistent superovulatory response in beef cattle following two intramuscular injections of FSH, without adversely affecting the number of transferable embryos.

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## Ovarian follicle reserve: emerging concepts and applications

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### Abstract

This paper presents new concepts in the study of folliculogenesis and describes some of the current applications to reproductive biotechnology. The importance of better understanding this issue is addressed both for basic and applied research. After a brief review of the basic conceptions of the origin, formation, and growth of follicles according to established concepts, some controversial points, as the postnatal production of the follicles and the role of multiocyte follicles, are discussed. The importance of the ovarian follicular reserve is considered for fertility and reproductive parameters, as well as some questions about the presence of multiocyte follicles in adult ovaries. Finally, some future prospects are proposed.

**Keywords:** follicular population, folliculogenesis, multiocyte follicle, neo-folliculogenesis, preantral follicle.

### Introduction

The interest in both applied and basic issues relating to folliculogenesis has increased significantly in the recent years. Many points may clarify this increased interest. In commercial aspects, the industry of embryo production, most notably *in vitro* embryo production, has dramatically increased worldwide. Other biotechnologies, such as timed artificial insemination, cloning and transgenic animals have been widely used, due to their benefits on animal breeding or human health. However, many aspects in follicular physiology remain unknown, particularly the role of the ovarian follicle reserve and fertility in cattle. Regarding basic research, some new theories have been presented on the follicular pool, providing interesting debates about follicle origin and the mechanisms involved in their recruitment and growth. The purpose of this article is to summarize recent studies focusing on how the follicle reserve is related to the improvement of reproductive efficiency

### Basic concepts of folliculogenesis

In female mammals, folliculogenesis starts during fetal life (Fig. 1). First, primordial germ cells

migrate from the yolk sac to the primordial gonads. Initially by mitosis, these germ cells are multiplied and many groups of oogonia are established, interacting one to each other by cytoplasmic communications. After that, oogonia are surrounded by somatic cells, forming the cortical cords, which are precursors of primordial follicles. The oogonias differentiate into oocytes, which will form primordial follicles when associated with pregranulosa cells. Oocytes initiate meiotic division and arrest at the prophase of meiosis I, in diplotene stage. This interruption lasts until follicle recruitment, either in puberty or final period of the reproductive life (Soto-Suazo and Zorn, 2005; van den Hurk and Zhao, 2005).

### Antral follicles, number of oocytes, and embryo production

The impact of the size of the ovarian follicle reserve on applied aspects of reproduction, such as fertility and pregnancy rates has challenged the conventional concept of the ovary as a fairly static organ. There is high variability among individual animals in the numbers of preantral and antral follicles and oocytes in the ovaries of bovine females, and it seems that the breed and or subspecies may have a strong influence on that, especially when comparing *Bos taurus* vs. *Bos indicus* (Burns *et al.*, 2005; Ireland *et al.*, 2007; Santos *et al.*, 2012). However, there is repeatability in antral follicle count (AFC) within individuals regardless of age, breed, stage of lactation or season (Burns *et al.*, 2005). This repeatability was also observed on *Bos indicus-taurus* females (Santos *et al.*, 2012).

Extreme variation among donors in embryo production by *in vitro* and *in vivo* methods remains one of the problems in bovine embryo production. It is interesting to note that some donors present better results following MOET or IVF, independently of the number of follicles and oocytes (Pontes *et al.*, 2009). However, the most common situation is a higher production of embryos from those donors with higher number of follicles (Table 1).

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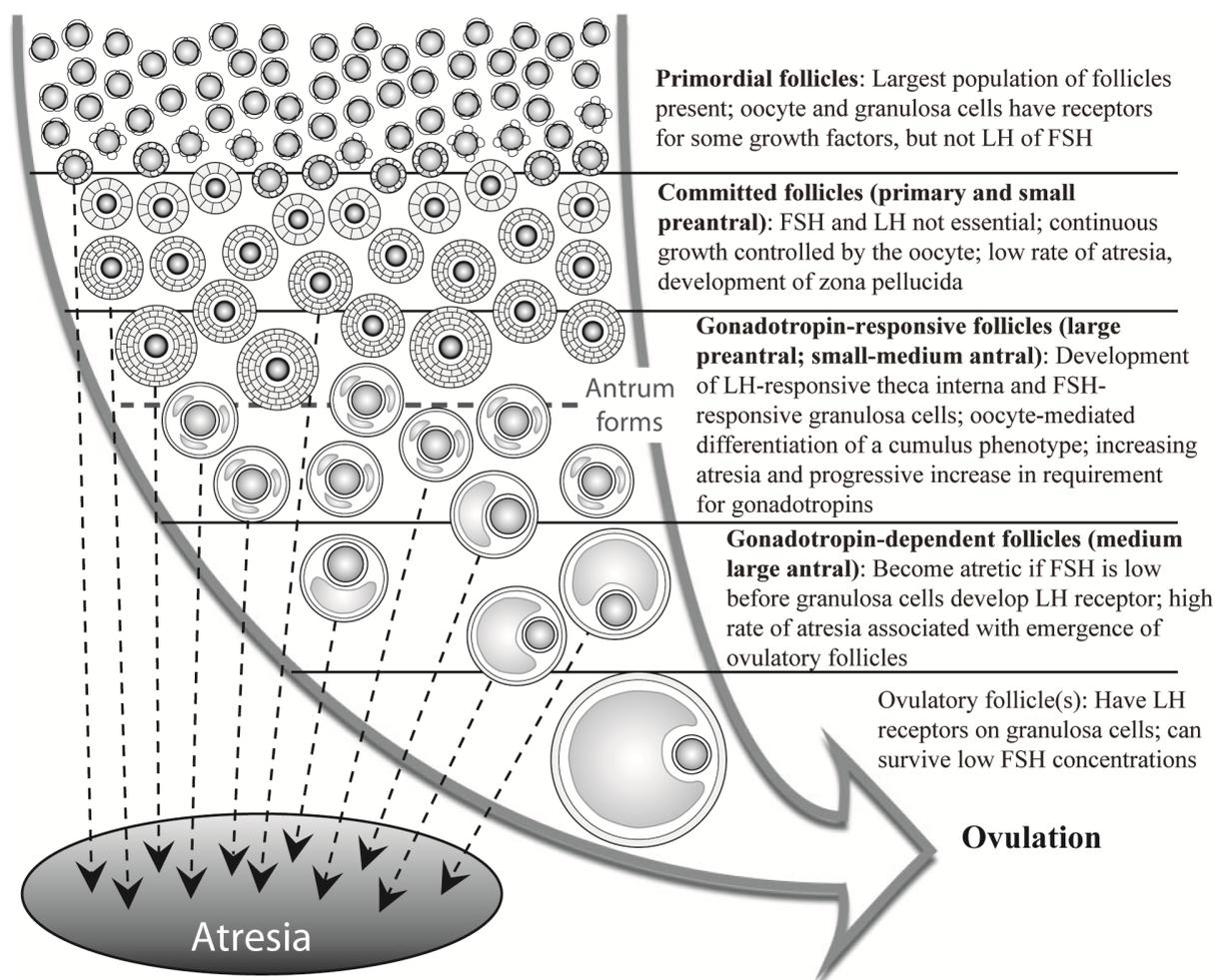


Figure 1. A model of folliculogenesis in ruminants. Adapted from Scaramuzzi *et al.* (2011).

Table 1. Variation in embryo production among 6 Nelore cows (I-VI), comparing *in vitro* (OPU/IVF) versus *in vivo* (MOET) procedures.

	Donors (I-VI)					
	I	II	III	IV	V	VI
Total no. OPU IVF cycles	5	5	4	4	5	5
Mean no. oocytes/collection	36.6	25.6	49	29.7	22.8	16
Mean no. viable oocytes/collection	32.2	23.4	45.2	26	19.6	14.4
Mean no. embryos/OPU IVF	15.6	10.4	24.1	10.3	6.8	3.8
Mean no. pregnancies/OPU IVF	4.8	2.8	9.25	4.3	2.2	1
Total no. MOET cycles	2	3	2	2	2	3
Mean no. embryos/collection	10	4.3	6.5	2	12.5	5.3
Mean no. pregnancies/collection	5.5	2	1	1.5	6.5	1.3

From Pontes *et al.* (2009).

In large scale commercial embryo production programs, individual animal variation in the number of follicles is very important (Table 2). The ordinary method of donor selection in this case consists in performing a pre-evaluation by ultrasound of the

ovaries, trying to identify those animals with higher numbers of antral follicles. It is important to emphasize that this selection, based on number of follicles, must be done after the evaluation of the genetic merit of the donors.



Table 2. Mean ( $\pm$  SD) for reproductive performance following OPU/IVP procedures ( $n = 656$ ) performed in Nelore donors ( $n = 317$ ), sorted according to oocyte collection (G1 = highest production and G4 = lowest production). Values given are per donor animal.

Group	No. cattle	No. viable oocytes	No. viable embryos	No. pregnancies on day 30	No. pregnancies on day 60
G1	$n = 78$	$47.06 \pm 1.6^a$	$15.06 \pm 0.86^a$	$5.62 \pm 0.54^a$	$5.52 \pm 0.81^a$
G2	$n = 80$	$24.95 \pm 0.33^b$	$9.17 \pm 0.63^b$	$3.63 \pm 0.36^b$	$3.32 \pm 0.33^b$
G3	$n = 79$	$15.57 \pm 0.26^c$	$6.00 \pm 0.39^c$	$2.10 \pm 0.21^c$	$1.92 \pm 0.20^b$
G4	$n = 80$	$6.31 \pm 0.38^d$	$2.42 \pm 0.25^d$	$0.92 \pm 0.13^d$	$0.85 \pm 0.13^c$
Total	$n = 137$	$23.35 \pm 0.72$	$8.13 \pm 0.30$	$3.03 \pm 0.15$	$2.91 \pm 0.013$

<sup>a,b,c,d</sup>Within a column, means without a common superscript differed ( $P < 0.05$ ). From Pontes *et al.* (2011).

### Fertility and number of antral follicles

In recent years, low AFC in dairy cattle has been associated with some characteristics of low fertility, such as smaller ovaries, lower numbers of follicles and oocytes in the ovaries (Ireland *et al.*, 2008), lower odds of being pregnant in the end of the breeding season (Mossa *et al.*, 2012), reduced responsiveness to superovulation (Singh *et al.*, 2004; Ireland *et al.*, 2007), lower circulating concentrations of progesterone and anti-Müllerian hormone (AMH; Ireland *et al.*, 2008; Jimenez-Krassel *et al.*, 2009), reduced endometrial thickness from day 0 to day 6 of the estrous cycle (Jimenez-Krassel *et al.*, 2009), and higher amounts of cumulus cell markers for diminished oocyte quality (Ireland *et al.*, 2009).

Nutritional influences affect folliculogenesis at multiple levels (Scaramuzzi *et al.*, 2011). Increased energy supply is known to exert a stimulating effect at an ovarian level (Letelier *et al.*, 2008). Despite the fact that some dietary components like IGF-I or leptin are great stimulators of follicular growth (Muñoz-Gutiérrez *et al.*, 2005; Mihm and Evans, 2008), it is unlikely that there is a single metabolic mediator of nutritional influences on folliculogenesis.

In cattle suffering food restriction in the first trimester of pregnancy, the number of antral follicles of the fetus can be reduced up to 60%, although the weight of the calf at birth was not altered (Mossa *et al.*, 2013). Furthermore, in sheep variations in maternal diet can not only limit the growth of the fetus, but also inhibit the expression of genes related to the release of pituitary gonadotropins. The decrease in FSH and LH levels may in turn result in a reduction of the number of follicles

and delayed fetal ovarian development (Borwick *et al.*, 1997; da Silva *et al.*, 2002).

The health management of cattle on dairy farms influences long-term reproductive performance of the herd. Mammary gland infection is the major factor affecting somatic cell count (SCC) in milk. Studies suggest that offspring of cows with chronically high SCC during gestation have reduced anti-Müllerian hormone (AMH) concentrations. Lower AMH concentrations are indicative of a diminished size of the ovarian reserve and potential reduction in reproductive efficiency (Ireland *et al.*, 2010; Evans *et al.*, 2012).

### Population of antral follicles versus ovarian reserve

It is known that *Bos indicus* cattle have 3-4 times more antral follicles and number of oocytes than *Bos taurus* cattle (Pontes *et al.*, 2009, 2011). To test if the ovarian reserve could account for this difference, we counted preantral follicles in ovaries obtained at abattoirs from fetuses, heifers and cows (Nelore and Aberdeen Angus). There was no clear answer to explain why *Bos indicus* donors have more antral follicles and produce more oocytes than *B. taurus* females. Despite the high variation between groups and among females within the same group, there were no differences between the number of preantral follicles in the ovaries of fetuses, heifers or cows of Nelore versus *Bos taurus* females (Fig. 2). These results suggest that total follicle reserve is not the reason for the difference in oocyte yield (Silva-Santos *et al.*, 2011). We are currently investigating the rate of follicular atresia in Nelore cows and the possibility of it being lower in *indicus* than in *taurus* females.

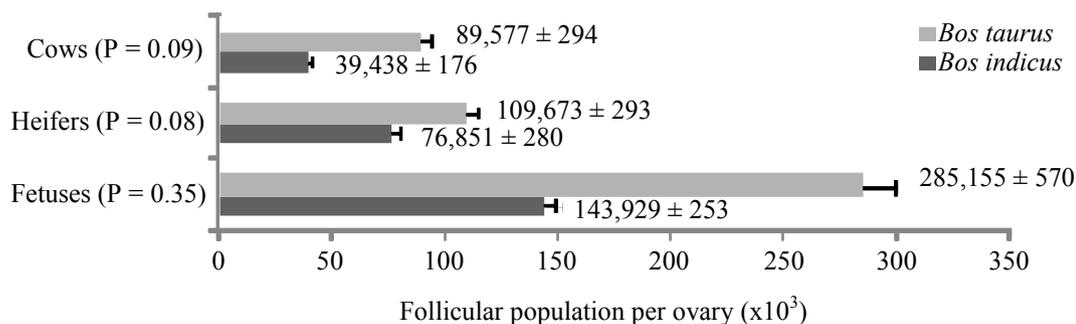


Figure 2. Average number of preantral follicles in the ovaries of *Bos indicus* (Nelore) and *Bos taurus* (Angus) females (mean ± SEM). From Silva-Santos *et al.* (2011).

### Molecular markers for the ovarian reserve

Inter-individual variability in oocyte production and in the response to exogenous ovarian stimulation remains a main limiting factor for embryo production in cattle. Thus, predictive tools for early selection of potentially high oocyte/embryo producing cattle are of critical importance in embryo programs. AMH, a member of the transforming growth factor- $\beta$  family produced by granulosa cells from healthy growing follicles, is currently recognized as a good marker of the ovarian reserve status and represents a good predictor of ovarian response to superstimulation (Monniaux *et al.*, 2010; Rico *et al.*, 2012). AMH concentration is correlated with the number of follicles recruited into the follicular waves during estrous cycles and is used as a biomarker of the follicle population in cattle. It was shown that cattle with high AFC (>25 follicles) presented higher circulating AMH concentrations compared to cattle with low AFC (<15 follicles;  $P < 0.01$ ) and a high correlation between the average AMH concentration and the average peak AFC was seen ( $r = 0.88$ ,  $P < 0.001$ ; Ireland *et al.*, 2008). Moreover, AMH concentrations vary minimally during oestrous cycles of beef and dairy cows (Ireland *et al.*, 2008; Rico *et al.*, 2009) and during menstrual cycles in women (Hehenkamp *et al.*, 2006; La Marca *et al.*, 2006), and has the same level of accuracy and clinical value as AFC to predict the response in assisted reproduction therapy (ART; Hendriks *et al.*, 2005, 2007; Broer *et al.*, 2009).

Considering the relationship of AMH with embryo production and antral follicle count, it is interesting to note that a single ultrasound evaluation presents a good picture of the number of antral follicles recruited per wave in that animal, in all reproductive cycles. This way, we can think about taking the ovary scanning as a possible alternative to predict the AMH level.

### The neo-folliculogenesis debate

For more than a century, it has been generally accepted that oocytes cannot be renewed in postnatal life. According to this concept, the number of oocytes is permanently defined in fetal ovaries (Zuckerman, 1951).

Nevertheless, in the last decade, this assumption has been in discussion. A group reported the presence of specific markers of meiosis in the ovaries of adult mouse females (Johnson *et al.*, 2004). Giving the current concept, such event should only take place during the fetal phase. A year later, female mice were subjected to chemical sterilization, with reports of the absence of follicles after the treatment. The same animals received a transfusion of bone marrow and peripheral blood and, a week later, viable follicles were identified in the ovaries (Johnson *et al.*, 2005).

The hypothesis of Johnson *et al.* (2004, 2005) has been strongly debated and some aspects of the experiments were heavily criticized by other researchers. Eggan *et al.* (2006) investigated the follicular renewal from cells spread through the blood in parabiotic mice (animals with shared bloodstream). Since one of the females was transgenic for a fluorescent protein, it was assumed that fluorescent oocytes should be found in the ovaries of the other female. However, even after repeated trials, the results have been contradictory to the neo-folliculogenesis theory.

Indirectly, in accordance with the theory of postnatal follicular renewal, Dyce *et al.* (2006) isolated follicle-like structures from porcine skin-derived fetal stem cells. These structures were capable of producing steroid hormones and were responsive to gonadotropins, in addition to producing embryo-like structures by parthenogenesis. Conversely, Liu *et al.* (2007) searched adult human ovaries for the presence of key genes involved in meiosis. No evidence of the occurrence of meiosis was found, contradicting the hypothesis of follicular renewal.

More recently, Kerr *et al.* (2012) monitored the number of primordial follicles throughout postnatal life and following depletion of the primordial follicle reserve, reported no indication of follicular renewal. Accordingly, Zhang *et al.* (2012) traced the development of female germline cell lineage and found no mitotically active female germline progenitors in postnatal ovaries. On the other hand, somatic cell generated from differentiating embryonic stem cells (ESCs) expressed specific granulosa cell markers. When



injected into neonatal mouse ovaries, these cells became incorporated within the granulosa cell layer of immature follicles and were able to synthesize steroids and respond to FSH (Woods *et al.*, 2013). Showing new data in the neo-folliculogenesis theory, Tilly's group described a protocol for isolation of female germline stem cells from adult ovarian tissue, along with cultivation and characterization of these cells before and after ex-vivo expansion (Woods and Tilly, 2013).

Independently of the individual opinion on the matter, we consider that the discussion is valuable, since new information has been added on the knowledge of follicular reserve.

### Multioocyte follicles

Follicles containing two or more oocytes have been described in the ovaries of adult females of several mammalian species (reviewed by Silva-Santos and

Seneda, 2011). These structures have been called multioocyte follicles (Fig. 3) and their contribution to ovulation and fertility in adult females is not currently known. Follicles with more than one oocyte have been well documented during fetal development. In this period, the ovarian cords are formed, which are tube-like structures containing germ cells surrounded by pregranulosa cells (Juengel *et al.*, 2002). However, the presence of these structures in adult ovaries is an intriguing physiologic phenomenon. It remains to be determined if these multioocyte follicles are simply remaining structures of the fetal phase or if they could be active and have a roll in an unknown pattern of follicle development in adult females. The presence of multioocyte follicles in *Bos indicus* females is particularly intriguing, considering the higher number of antral follicles and the same number of preantral follicles when comparing *Bos taurus* and *Bos indicus* ovaries (Silva-Santos *et al.*, 2011).

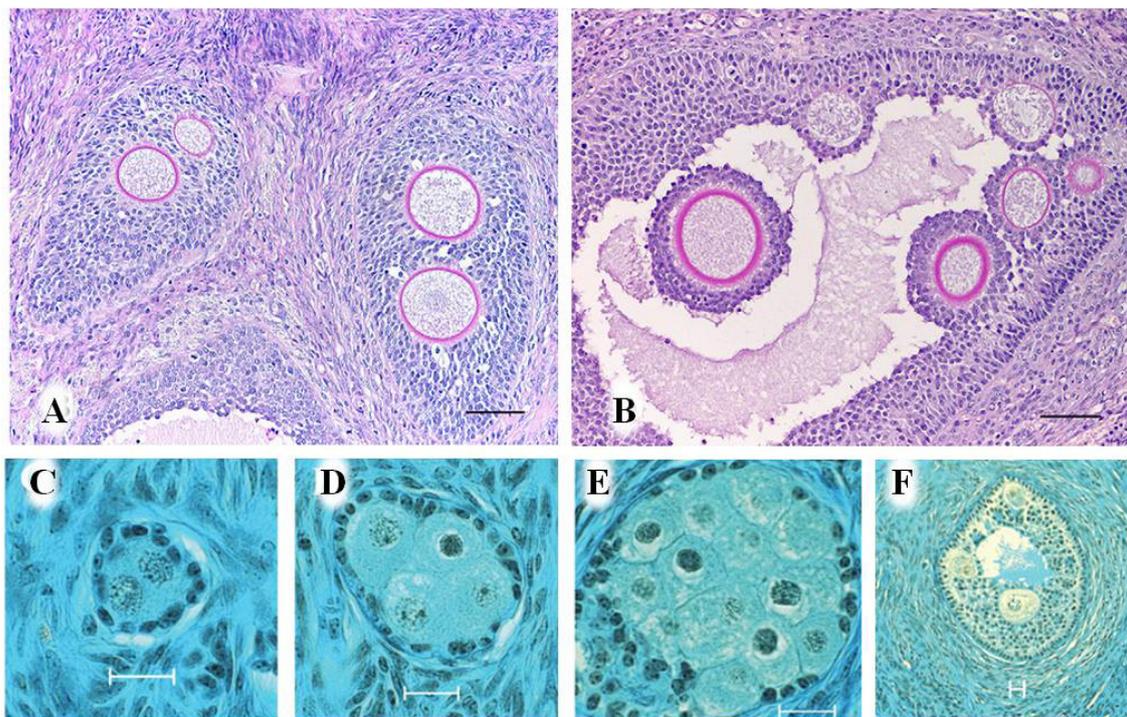


Figure 3. Preantral and antral multioocyte follicles in mature bitch (A-B) and heifer (C-F) ovaries. Multioocyte follicle at the secondary stage of development with two oocytes in each follicle (A), at the antral stage with six oocytes (B), at the primary stage with two (C), four (D) and 13 oocytes (E), and at the antral stage with three oocytes (F). Scale bars: 70  $\mu$ m. Adapted from Ireland *et al.* (2008); Payan-Carreira and Pires (2008).

### Conclusions and future remarks

As briefly described, folliculogenesis remains as a universe to be explored. Despite the extraordinary progress we have seen in the last years, there are several gaps to be filled, especially in the preantral phase. Neo-oogenesis can still be considered as a hypothesis. However, it seems reasonable to discuss that the follicle

formation may have new concepts not understood so far. For instance, we need to improve our knowledge about the multioocyte follicles and the follicular cords in adult females. Also, the number of oocytes obtained from *indicus* cows remains unclear at this moment, since the number of preantral follicles in Zebu females is similar to the amount observed in *taurus* animals.

Cattle can be selected based on counting antral



follicles using ultrasonography. Its use in the field is stimulating, since ultrasonography is an easy and fast tool that can be applied and that should not alter management conditions. Therefore, more oocytes/embryos per donor may be obtained. However, the genetic impact of selecting cattle with high AFC remains to be considered, especially taking into account the large number of descendants that can be produced by IVF from a single donor.

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## Equine preantral follicle harvesting, processing, and *in vitro* culture: the journey has already started

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### Abstract

Preantral follicles are of great abundance in mammalian ovaries and the vast majority (>99.9%) never become ovulatory; therefore, the ability to rescue these otherwise wasted follicles seems very appealing. Considering there are striking similarities in antral follicle dynamics between mares and women, the mare might become a good model to study early (preantral and antral) folliculogenesis in women, with several advantages related to using an animal model. Studies in our laboratory recently validated the use of a transvaginal, ultrasound-guided ovarian Biopsy Pick-Up (BPU) method to harvest preantral follicles using the mare as a model to study early folliculogenesis (Haag *et al.*, 2013a, b, c). This article will review some of the important findings of our recent studies related to the harvesting, processing, and culture of equine preantral follicles and discuss those with the limited information available in the literature.

**Keywords:** biopsy pick-up, equine, folliculogenesis, ovary, preantral follicle.

### Introduction

The importance of research involving preantral follicles has become more apparent in recent decades. Oocytes are in great abundance in the mammalian ovary, yet only a very small portion (<0.01%) of these oocytes are ever released from preovulatory follicles via ovulation and have a chance to be potentially fertilized. A vast majority of oocytes are enclosed within preantral follicles which will either die by the process of follicular atresia at some point during development or never become activated and remain dormant. Therefore, the ability to rescue these otherwise wasted follicles is very attractive.

The development of technologies to harvest and isolate preantral follicles and mature them *in vitro* holds many promising applications because of the great abundance of preantral follicles in the mammalian ovary. Cryopreservation and/or *in vitro* culture of preantral follicles could potentially serve many purposes, such as large-scale embryo production from individuals with high genetic merit, establishment of gamete banks for rare or endangered species, advancement of knowledge for contraception purposes in wild animals, development of bioassays to test toxic

effects of pharmaceutical and environmental agents, and preservation of fertility in humans whose preantral follicle population has been jeopardized by chemotherapy and/or radiotherapy cancer treatments, which partially or entirely eliminate the follicle reserve (Picton *et al.*, 2000). The success of these technologies is completely dependent upon understanding the specific mechanisms that regulate follicle and oocyte growth and development. The amazing benefits that could potentially be provided through *in vitro* culture of preantral follicles make the understanding of early folliculogenesis a top priority research area. During the past 10 yr, the field of ovarian folliculogenesis has seen a large amount of focus placed upon the study of preantral follicles. Although there is limited knowledge of the mechanisms that control preantral follicle dynamics, researchers are beginning to understand how preantral follicles undergo activation and growth through *in vitro* culture studies.

In light of the fact that there are several noteworthy similarities between women and mares regarding antral follicle dynamics (Ginther *et al.*, 2004, 2005; Baerwald, 2009; Gastal, 2009, 2011; Ginther, 2012), the mare could potentially become an appropriate model for studying early folliculogenesis in women, with an animal model being advantageous in numerous ways. Obtaining material to be used for *in vitro* culture can be difficult, mainly because for several species the only sources of preantral follicles are slaughterhouse or ovariectomized ovaries. This problem is even more amplified in mares, especially in the United States where accessible ovarian tissue is very scarce due to the closing of all equine abattoirs in 2007. Hence, a transvaginal, ultrasound-guided ovarian biopsy procedure that would allow for the repeated collection of preantral follicles *in vivo* could be very beneficial in providing material for the study of early ovarian folliculogenesis *in vitro* (Lass *et al.*, 1997; Aerts *et al.*, 2005).

In order to expand upon the limited understanding of early folliculogenesis in the equine species, the following hypotheses were tested in our recent studies (Haag *et al.*, 2013a, b, c): 1) the transvaginal, ultrasound-guided Biopsy Pick-Up (BPU) method provides sufficient material for studies on the early stages (primordial, transitional, and primary follicles; Fig. 1) of folliculogenesis in mares; 2) preantral follicle quantity, viability, and morphology do not differ according to phase of the estrous cycle; 3) younger mares have more follicles per mg of tissue than

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older mares; 4) rate of atresia increases with follicle class; 5) rate of morphologically normal follicles and follicle class proportions are similar between tissue chopper (mechanical isolation) and histological analyses; 6) rate of morphologically normal follicles, follicle viability, and follicle class proportions are similar between follicles from *in vitro* BPU fragments and scalpel blade dissected fragments; 7) a substantial number of follicles are lost during mechanical isolation using a tissue chopper compared to *in situ* histological analysis, regardless of the methodology

used for harvesting tissue (i.e. *in vitro* BPU vs. scalpel blade); 8) preantral follicles submitted to an *in vitro* culture system will respond positively by undergoing activation and growth while remaining morphologically normal; and 9) preantral follicles will respond differently depending on the base medium ( $\alpha$ -MEM or TCM-199) used for *in vitro* culture. This article will review some of the important findings of our recent studies related to the harvesting, processing, and culture of equine preantral follicles and discuss those with the limited information available in the literature.

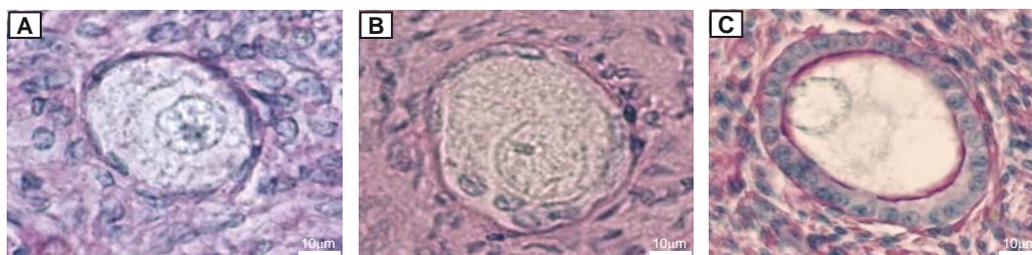


Figure 1. Morphology of equine preantral follicles. Normal (A) primordial, (B) transitional, and (C) primary follicles.

### Early folliculogenesis

Folliculogenesis, or the complete development of ovarian follicles and their respective oocytes from the early primordial phase through complete maturation, is a vital feature of female fertility and reproduction in general. However, the mechanisms that control this phenomenon are not well understood, especially during the earlier stages of folliculogenesis. Recent technologies, including *in vitro* preantral follicle culture, are beginning to give insight into how early follicle activation and growth occur.

It has long been accepted that female mammals are born with a finite pool of resting follicles which constitute the ovarian reserve (Gougeon, 2010). However, recent research suggests that the mammalian ovary may be capable of generating new oocytes after birth (White *et al.*, 2012). Regardless, the follicle population decreases with age as the follicles enter into the growth phase, or the more likely result, undergo follicular atresia (Eppig and O'Brien, 1996). Primordial (one layer of flattened granulosa cells around the oocyte), transitional (both flattened and cuboidal granulosa cells around the oocyte), and primary (one layer of cuboidal granulosa cells around the oocyte) follicles make up a large majority of the total ovarian reserve (Silva *et al.*, 2004b). The ovarian follicle population differs greatly among species, but seems to follow the same trend as the population reaches its peak during fetal life, diminishes greatly by parturition, and continues to be depleted well into adult life. Rat ovaries are estimated to contain 64,000 normal follicles/oogonia at 17.5 days post-conception, 39,000 at birth, and 19,000 by 2 days post-partum (Beaumont and Mandl, 1962). A similar trend is seen in humans where the total follicle/oocyte population reaches its peak of 6,800,000

at 5 months post-conception, drops to 2,000,000 at birth, and reaches 300,000 by 7 yr post-partum (Baker, 1963; Wise *et al.*, 1996). Preantral follicle populations have not been studied in the fetal or neo-natal horse ovary, but the ovaries of 2- to 4-yr-old pony and saddle-type mares contain an average of 35,590 primordial follicles (Driancourt *et al.*, 1982). Since mares only naturally ovulate an average of 12.4 times per year (Kot and Tischner, 1991), it is obvious that a huge majority (>99%) of primordial follicles from the initial ovarian reserve will never reach the ovulatory phase.

Very little is known about what causes preantral follicles to enter the growth phase. Studies showing that early folliculogenesis is normal in women with follicle-stimulating-hormone (FSH) deficiency (Kumar *et al.*, 1997) and in both FSH- $\beta$  subunit (Matthews *et al.*, 1993) and FSH-receptor knock-out (KO) mice (Dierich *et al.*, 1998) suggest that this process is gonadotropin independent. However, receptors for FSH have been identified in the granulosa cells of primary, secondary, and antral follicles in cattle (Wandji *et al.*, 1992) and hamsters (Roy and Albee, 2000), and in oocytes (Méduri *et al.*, 2002; Durlej *et al.*, 2011) of primordial and primary follicles and granulosa cells of primary follicles in swine (Durlej *et al.*, 2011). Therefore, it is possible that FSH can directly and/or indirectly stimulate primordial follicle growth by causing the release of various factors from later stage follicles or stroma cells that act in a paracrine fashion, such as IGF-I and activin (van den Hurk and Zhao, 2005). Several other molecules have been suggested to either inhibit or stimulate preantral follicle growth (for review see Monget *et al.*, 2012), but the specific details of this process remain unknown.



### Summary of recent equine preantral follicle studies

For a better understanding, and to facilitate the discussion of our results with the pertinent available literature, presented below are brief summaries of the results from our studies on equine preantral follicles (Haag *et al.*, 2013a, b, c).

#### *Study 1. Quantification, morphology, and viability of equine preantral follicles obtained via the biopsy pick-up method*

Due to the difficulty of obtaining equine ovarian tissue, a method for repeated collection of preantral follicles was tested in mares (Haag *et al.*, 2013c). The goals of this study were to refine the transvaginal, ultrasound-guided BPU method for ovarian stroma in mares and to assess the number, viability, and morphology of preantral follicles harvested. A total of 33 ovarian biopsy procedures were performed on 18 mares during the breeding season. Mares were 5- to 21-yr-old and biopsies were performed during the estrous and/or diestrous phase as confirmed by transrectal ultrasonography. Follicles were isolated mechanically using a tissue chopper, counted and classified, measured for follicle and oocyte diameter, and analyzed for either viability or morphology. A total of 256 biopsy attempts ( $n = 8$  biopsy attempts per biopsy procedure) were made resulting in 185 successful tissue sample collections (72% success rate). The mean weight of ovarian tissue collected per procedure was 25 mg. Overall, 620 preantral follicles were collected and isolated (95% primordial and 5% primary; Table 1). An average of 19 follicles were isolated per biopsy procedure. Primordial and primary follicles had an average diameter of 31 and 42  $\mu\text{m}$ , respectively. Viability rate (as assessed using Trypan Blue dye) was higher for primordial follicles (91%) compared to primary follicles (50%). Primordial follicles tended to have a higher rate of morphological normality (96%) compared to primary follicles (80%). The total number of follicles isolated, amount of tissue harvested, and number of follicles per mg of tissue did not differ according to phase of the estrous cycle (Table 2). Younger mares (ages 5 to 7 yr) had more follicles isolated per procedure than older mares (ages 14 to 21 yr). The length of the

interovulatory interval was not affected by any biopsy procedure, and no adverse effects in cyclicity or general reproductive health were observed. We concluded that the BPU method provided satisfactory numbers of normal and viable preantral follicles for the study of early follicular development in the equine species.

#### *Study 2. Equine preantral follicles obtained via the biopsy pick-up method: histological evaluation and validation of a mechanical isolation technique*

The purposes of this study (Haag *et al.*, 2013a) in mares were to 1) compare preantral follicle parameters between *in vitro* BPU and scalpel blade dissection collection methods and between histological and mechanical isolation (tissue chopper) processing techniques (Experiment 1); 2) histologically evaluate preantral follicles (Experiment 2); and 3) compare histological analysis with a previously established mechanical isolation technique for ovarian cortical fragments obtained *in vivo* using a BPU instrument (Experiment 3). For Experiment 1, a total of 220 preantral follicles were analyzed (90% primordial and 10% primary). Proportions of primordial and primary follicles did not differ between tissue collection (BPU vs. scalpel blade dissection) or processing (mechanical isolation vs. histology) methods. Follicle viability and morphological normality rates were similar between tissue collection methods (Fig. 2). For Experiment 2, a total of 332 preantral follicles were analyzed. Primordial and transitional (combined) follicles and oocytes averaged 36 and 26  $\mu\text{m}$  in diameter, respectively, whereas primary follicles and oocytes averaged 43 and 32  $\mu\text{m}$  in diameter, respectively. For Experiment 3, a total of 188 preantral follicles were analyzed. The proportion of primordial versus primary follicles was higher for histological analysis (98%) compared to tissue chopper analysis (94%) within the same animals. Number of follicles per mg of tissue was not different within animals when the processing methods were compared. We concluded that most parameters evaluated for preantral follicles were similar between histological and tissue chopper processing techniques, indicating that mechanical isolation is an efficient way to dissociate preantral follicles from the equine ovarian cortex.

Table 1. Diameter and number of follicles and oocytes harvested per BPU procedure.

	Primordial follicles (n)	Primordial follicle diameter ( $\mu\text{m}$ ) <sup>‡</sup>	Primordial oocyte diameter ( $\mu\text{m}$ ) <sup>‡</sup>	Primary follicles (n)	Primary follicle diameter ( $\mu\text{m}$ ) <sup>‡</sup>	Primary oocyte diameter ( $\mu\text{m}$ ) <sup>‡</sup>	Total preantral follicles (n)
Mean $\pm$ SEM	17.8 $\pm$ 1.7 <sup>a</sup>	31.0 $\pm$ 0.5 <sup>c</sup>	27.6 $\pm$ 0.6 <sup>c</sup>	1.0 $\pm$ 0.2 <sup>b</sup>	42.3 $\pm$ 1.6 <sup>d</sup>	34.4 $\pm$ 0.7 <sup>f</sup>	18.8 $\pm$ 1.9
Total (n)	588	277	277	32	11	11	620
% of total	94.8			5.2			100

<sup>‡</sup>Diameter was measured on morphologically normal follicles/oocytes only. <sup>a,b,c,d,e,f</sup>Same end points between primordial and primary follicles/oocytes without a common superscript differed ( $P < 0.05$ ). Adapted from Haag *et al.* (2013c).

Table 2. Mean ( $\pm$  SEM) diameter, total number, viability, and morphology of preantral follicles harvested per BPU procedure.

	Primordial follicle diameter ( $\mu\text{m}$ ) <sup>†</sup>	Primary follicle diameter ( $\mu\text{m}$ ) <sup>‡</sup>	Total preantral follicles (n) <sup>§,‡</sup>	Viability (% live) <sup>‡</sup>	Morphology (% normal) <sup>‡</sup>
<b>Age groups</b>					
Younger (6.0 $\pm$ 0.3 yr, n=6)	30.5 $\pm$ 0.7 <sup>a</sup>	38.2 $\pm$ 1.9	26.6 $\pm$ 4.7 <sup>a</sup>	90.7 $\pm$ 2.6 <sup>a</sup>	97.9 $\pm$ 1.9 <sup>a</sup>
Older (16.0 $\pm$ 0.8 yr, n=11)	31.0 $\pm$ 1.1 <sup>a</sup>	44.9 $\pm$ 3.9	15.5 $\pm$ 2.8 <sup>b</sup>	82.9 $\pm$ 4.4 <sup>a</sup>	94.0 $\pm$ 3.4 <sup>a</sup>
<b>Phase of estrous cycle groups</b>					
Estrous (n=15)	30.0 $\pm$ 0.8 <sup>a</sup>	40.6 $\pm$ 2.2	15.9 $\pm$ 2.6 <sup>a</sup>	91.1 $\pm$ 3.7 <sup>a</sup>	96.0 $\pm$ 1.9 <sup>a</sup>
Diestrous (n=18)	31.8 $\pm$ 0.9 <sup>a</sup>	41.7 $\pm$ 2.5	21.3 $\pm$ 3.4 <sup>a</sup>	87.6 $\pm$ 2.5 <sup>a</sup>	94.9 $\pm$ 2.5 <sup>a</sup>

<sup>†</sup>Number of primordial follicles evaluated ranged from 67 to 117 (age groups) and 116 to 157 (phase of estrous cycle groups). <sup>‡</sup>Not analyzed due to the low number of observations (n = 2 to 6). <sup>§</sup>Includes primordial and primary follicles. The total number of preantral follicles evaluated for younger versus older mares was 239 and 170, respectively. <sup>‡</sup>Comparison between estrous and diestrous phases was performed using 14 mares in consecutive phases of the estrous cycle. The number of preantral follicles evaluated was 223 and 298 for estrous and diestrous phases, respectively. <sup>a,b</sup>Within a column, values between age groups and between phase of the estrous cycle groups without a common superscript differed ( $P < 0.05$ ). Adapted from Haag *et al.* (2013c).

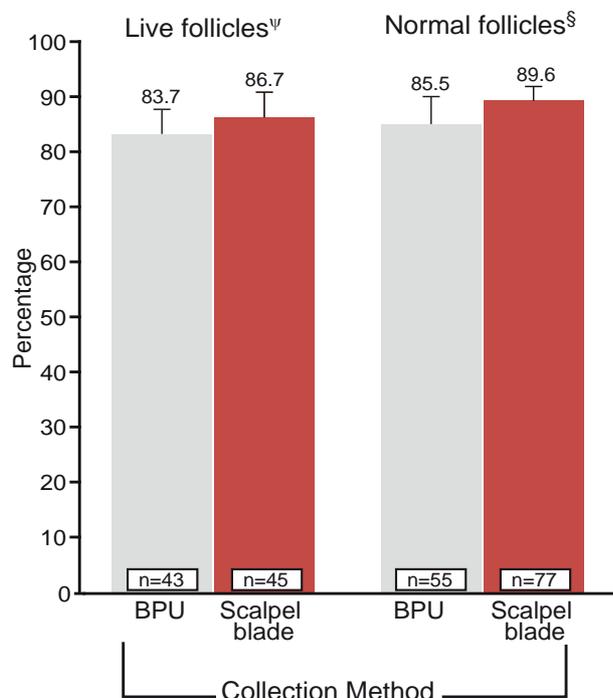


Figure 2. Overall viability and morphology rates comparing tissue collection (BPU vs. scalpel blade) methods. <sup>‡</sup>Data from tissue chopper analysis only. <sup>§</sup>Combined data from tissue chopper and histological analyses. Within each end point, collection methods did not differ ( $P > 0.05$ ).

### Study 3. *In vitro* culture of equine preantral follicles obtained via the biopsy pick-up method

The goals of this study (Haag *et al.*, 2013b) were to 1) study folliculogenesis in mares by developing

an *in vitro* culture system for preantral follicles obtained via the BPU method; and 2) determine which culture medium ( $\alpha$ -MEM or TCM-199) was most efficient for promoting follicular growth and development and maintaining morphologically normal follicles throughout



1 and 7 days of culture. Ovarian cortical strips were obtained from 5- to 16-yr-old mares ( $n = 10$ ) via the BPU method ( $n = 10$  procedures) during the breeding season. The base culture media were supplemented with glutamine, hypoxanthine, bovine serum albumin, insulin, transferrin, selenium, ascorbic acid, penicillin, and streptomycin and were named  $\alpha$ -MEM<sup>+</sup> and TCM-199<sup>+</sup>. Ovarian tissue was immediately submitted to histological analysis (noncultured control; D0) or cultured *in situ* for 1 day (D1) or 7 days (D7) in either  $\alpha$ -MEM<sup>+</sup> or TCM-199<sup>+</sup> and submitted to histological analysis, generating five treatment groups: noncultured control,  $\alpha$ -MEM:D1, TCM-199:D1,  $\alpha$ -MEM:D7, and TCM-199:D7. A total of 142 preantral follicles were analyzed in five replicates. No follicles were observed in the TCM-199:D7 treatment group. The proportion of primordial follicles was higher in the control group compared to the  $\alpha$ -MEM:D7 treatment group. The proportion of primary follicles was higher in the  $\alpha$ -MEM:D7 treatment group compared to the control. In addition, the proportion of developing follicles (transitional, primary, and secondary) was higher in the

$\alpha$ -MEM:D7 treatment compared to the control group (Fig. 3). These results indicate that follicular activation did occur. There was no difference in the percentages of normal primordial and primary follicles among treatments. A higher percentage of normal developing follicles was observed in the  $\alpha$ -MEM:D1 treatment compared to the TCM-199:D1 and  $\alpha$ -MEM:D7 treatment groups. Overall, the percentage of normal follicles was higher in the control (72%) and  $\alpha$ -MEM:D1 (84%) treatments compared to the  $\alpha$ -MEM:D7 (27%) treatment group. Mean follicle diameter was greater in the  $\alpha$ -MEM:D7 treatment (41  $\mu$ m) compared to the control group (37  $\mu$ m). Mean oocyte diameter (31-33  $\mu$ m) was greater in the  $\alpha$ -MEM:D1, TCM-199:D1, and  $\alpha$ -MEM:D7 treatments compared to the control group (27  $\mu$ m). We concluded, based on these preliminary data, that the *in vitro* culture of equine ovarian fragments obtained *in vivo* via the BPU method promoted preantral follicle development and follicle and oocyte growth in  $\alpha$ -MEM<sup>+</sup> for 7 days, with some follicles maintaining morphological normality throughout the culture period.

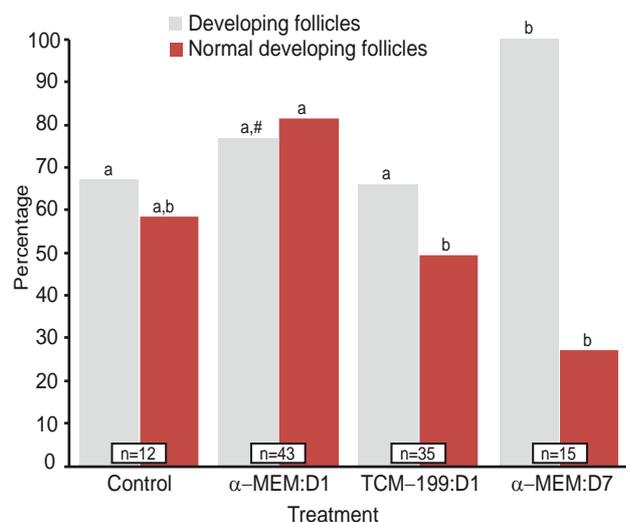


Fig. 3. Percentage and morphology of developing follicles in culture. Among treatment groups, bars for each end point without a common letter differed ( $P < 0.05$ ). #Tended ( $P < 0.06$ ) to differ from the  $\alpha$ -MEM:D7 treatment group. Only developing follicles were observed in the  $\alpha$ -MEM:D7 treatment group. No follicles were observed in the TCM-199:D7 treatment group.

#### Limitations to studying early folliculogenesis in the equine species

Folliculogenesis begins when resting follicles from the ovarian reserve are activated for growth and terminates when a follicle reaches the ovulatory phase. This process can be broken down into four basic steps (for review see Gougeon, 2010): 1) initiation; 2) early follicle growth; 3) follicle selection; and 4) maturation of the preovulatory follicle. Tools such as transrectal ultrasonography and follicle aspiration have allowed for

extensive studies on follicle selection and maturation of the preovulatory follicle in the equine species (for reviews see Gastal, 2009, 2011). However, these technologies are limited to the study of antral follicles. This barrier highlights the importance of introducing new technologies that allow for the study of early folliculogenesis in mares.

Having access to ovarian tissue is one of the main limiting factors in studying early folliculogenesis in any species. A majority of studies in the literature use preantral follicles that were harvested from whole ovaries



acquired from individuals that were slaughtered, euthanized, or died unexpectedly, or through ovariectomy. This presents a problem in the equine species, for which abattoirs are not as abundant as for other species.

### Biopsy Pick-Up method

In cases where whole ovaries are scarce or unavailable, the BPU method provides a good alternative, and even has some advantages. Whereas whole ovaries are only available from dead or ovariectomized individuals, the BPU method offers the advantage of being able to repeatedly harvest preantral follicles from live individuals, apparently without jeopardizing their short-term reproductive function or general health. This technology was originally introduced in humans as a means to evaluate the ovarian reserve of preantral follicles using ovarian cortical biopsies (Lass *et al.*, 1997). The BPU method has been used to harvest preantral follicles from women (Lass *et al.*, 1997; Meiorow *et al.*, 1999; Qu *et al.*, 2000; de Bruin *et al.*, 2002; Schmidt *et al.*, 2003; Rice *et al.*, 2008; Zhou *et al.*, 2010; David *et al.*, 2011) and cows (Aerts *et al.*, 2005, 2008). Studies in women have used ovarian cortical biopsies to evaluate the ovarian reserve of preantral follicles (Lass *et al.*, 1997; Schmidt *et al.*, 2003), to analyze cryopreservation techniques to preserve fertility (Meiorow *et al.*, 1999; Qu *et al.*, 2000; Zhou *et al.*, 2010), and to evaluate the ultrastructure of preantral follicles (de Bruin *et al.*, 2002). Preserving fertility in women whose resting follicle reserve is threatened by medical procedures such as chemotherapy and/or radiotherapy treatment is one of the ultimate goals associated with ovarian biopsy. Even though human preantral follicles from ovarian biopsy tissue have been successfully cryopreserved, indicated by no difference in follicle morphology before or after cryopreservation (Qu *et al.*, 2000), a preantral follicle culture system that can facilitate complete folliculogenesis has not been established in this species.

Aerts *et al.* were the first group to use the transvaginal BPU method to harvest preantral follicles from a live animal (cow) to evaluate the effects of BPU on post-mortem ovaries (Aerts *et al.*, 2005) and to quantify and analyze the viability of preantral follicles (Aerts *et al.*, 2008). These two bovine studies revealed that the vast majority (93%) of follicles analyzed were considered viable and suitable for culture. Additionally, a post-mortem analysis of the repeatedly biopsied ovaries revealed neither adhesions nor morphological abnormalities and no evidence of scar tissue formation. Regular ovarian activity continued after the biopsy procedure was performed as evidenced by ultrasonographic examination in live animals and the presence of several new antral follicles on post-mortem ovaries. While a post-mortem analysis of the biopsied

ovaries was not possible in our recent study (Haag *et al.*, 2013c), continued ultrasound scanning of the ovaries well after the BPU procedures was performed and revealed resumed normal cyclicity in all mares, with no change in interovulatory interval before, during, or after the BPU procedure(s). In addition, no visible changes in the ovaries were apparent during later ultrasound examinations. A gross and microscopic examination of biopsied mare ovaries would be very helpful in determining any possible short- or long-term effects of the BPU procedure on the ovaries.

Due to the closing of the last equine abattoir in the United States in 2007, ovarian tissue has been difficult to obtain in recent years for studies on early folliculogenesis in mares. Therefore, it is easy to realize the importance of the BPU method to get around this problem. Not only does the procedure provide adequate numbers of preantral follicles (Haag *et al.*, 2013a, b, c), but it can also be performed repeatedly on the same individuals. This could allow for future studies that analyze preantral follicle populations continuously throughout a mare's lifetime, studying the effect of age or other factors, such as pathologies, on the resting follicle reserve. The alternative approaches (ovaries obtained from dead or ovariectomized mares) for harvesting preantral follicles are not repeatable within individuals and would not allow for the same types of dynamic studies.

Not only does the BPU method provide satisfactory numbers of preantral follicles, but it also provides mostly morphologically normal and viable follicles, indicating their suitability for *in vitro* culture. The overall follicle viability rates observed in our recent studies (85.2 to 91.1%; Haag *et al.*, 2013a, b, c) are similar to those seen in cows for preantral follicles obtained via the BPU method (92.8%; Aerts *et al.*, 2008). A comparison between preantral follicles from *in vitro* BPU fragments and scalpel blade dissected fragments showed no difference in follicle morphology or viability according to collection method (Haag *et al.*, 2013a), suggesting that the BPU procedure itself does not affect those parameters.

### Preantral follicle isolation

In order to analyze preantral follicles (except for *in situ* histological analysis), they must first be isolated from the surrounding ovarian tissue. Preantral follicles have been released from the ovarian stroma of several species using either enzymatic (human: Roy and Treacy, 1993; bovine: Carámbula *et al.*, 1999; murine: Demeester *et al.*, 2002) or mechanical isolation (bovine: Nuttinck *et al.*, 1993; caprine: Lucci *et al.*, 1999a, b; ovine: Amorim *et al.*, 2000), or a combination of both methods (bovine: Figueiredo *et al.*, 1993). Our recent studies (Haag *et al.*, 2013a, c) are the first in the equine species to dissociate preantral follicles from the ovarian



stroma using a mechanical isolation technique.

In our recent studies (Haag *et al.*, 2013a, c), the mechanical approach was preferred over enzymatic isolation because of results from previous enzymatic isolation studies in this species. Apparently, only three studies exist in which equine preantral follicles were isolated from the ovarian stroma, and all three studies employed enzymatic isolation using collagenase (Telfer and Watson, 2000) or collagenase in combination with DNase (Szlachta and Tischner, 2000, 2004). Telfer and Watson (2000) reported that 70% of all follicles had diameters between 90 and 150  $\mu\text{m}$  after incubation with collagenase, and Szlachta and Tischner (2000, 2004) reported follicle diameters between 60 and 220  $\mu\text{m}$  after incubation with collagenase and DNase. These diameters indicate that most follicles isolated enzymatically were most likely beyond the primordial or primary stage of development, which averaged 31 and 42  $\mu\text{m}$  in diameter, respectively, in our recent studies. In addition, Telfer and Watson (2000) were able to isolate only a small number of preantral follicles (~30 to 60) per ovary. Since primordial follicles are considered to be the prime starting material for *in vitro* culture (Cortvrindt and Smitz, 2001), mechanical isolation was used in our studies (Haag *et al.*, 2013a, c) based on previous studies in other species that isolated high proportions of primordial follicles using a tissue chopper (bovine: Nuttinck *et al.*, 1993; caprine: Lucci *et al.*, 1999a, b; ovine: Amorim *et al.*, 2000). The tissue chopper has been utilized by several research groups around the world and is considered a practical and fast way to mechanically isolate large numbers of viable and morphologically normal preantral follicles.

In our recent studies (Haag *et al.*, 2013a, c), not only did mechanical isolation provide a large number of preantral follicles with a high proportion of primordial follicles, but also the vast majority of the follicles were morphologically normal and viable. A previous study which analyzed extracellular matrix proteins in bovine preantral follicles showed the presence of collagen fibers in the basement membrane of preantral follicles and led the authors to believe that a mechanical defense was provided by the collagen fibers, protecting the follicle from physical damage during the mechanical isolation process (Figueiredo *et al.*, 1995). This is extremely important as the success of future technologies involving the further use of preantral follicles depends on the ability to begin with healthy follicles. It should be noted, however, that mechanical isolation using a tissue chopper has been associated with a high percentage of preantral follicles lost during the process. In studies analyzing recovery rates for preantral follicles mechanically isolated from whole ovary slices by comparing the data with *in situ* histological analysis, overall recovery rates were 28, 35, and 46% (36% overall) for prepubertal, nonpregnant adult, and pregnant adult caprine ovaries, respectively (Lucci *et*

*al.*, 1999b), and 5, 28, and 26% (17% overall) for fetal, nonpregnant adult, and pregnant adult ovine ovaries, respectively (Amorim *et al.*, 2000). Although in our study (Haag *et al.*, 2013a) there was no statistical difference in the number of follicles per mg of tissue comparing tissue chopper and histological analyses within animals, the number of follicles per mg of tissue was 34% lower for tissue chopper analysis (recovery rate: 66%). A study using larger numbers of equine preantral follicles from whole ovary slices (not just BPU fragments) might reveal a statistical difference between the two processing methods.

Even though enzymatic isolation using collagenase showed no deleterious effect on follicle morphology in mares (Telfer and Watson, 2000), that study and the other two studies in mares (Szlachta and Tischner, 2000, 2004) did not evaluate follicle viability or oocyte quality, and oocyte quality has been shown to be negatively affected by collagenase treatment in cows (Wandji *et al.*, 1996). However, Aerts *et al.* (2008) used collagenase to enzymatically isolate bovine preantral follicles from ovarian fragments obtained via BPU and observed an overall follicle viability rate of 92.8%. Further studies are necessary to compare the effects of enzymatic versus mechanical isolation of preantral follicles from equine ovarian tissue.

### Histological analysis

Only a few studies have evaluated equine preantral follicles using histological analysis for the purposes of estimating ovarian follicle populations (Driancourt *et al.*, 1982), evaluating preantral follicle morphology after enzymatic isolation (Telfer and Watson, 2000), and studying the distribution, morphology, and ultrastructure of preantral follicles (Szlachta and Tischner, 2002). All these studies used hematoxylin and eosin for staining and used sectioning intervals of 10, 7, and 2  $\mu\text{m}$ , respectively.

In our recent studies (Haag *et al.*, 2013a, b), tissue was cut at a 10  $\mu\text{m}$  sectioning interval and was stained with Periodic-acid Schiff (PAS) and counterstained with hematoxylin. PAS was chosen for its superior ability to stain glycoproteins, which is the main component of the zona pellucida surrounding the oocyte. A 10  $\mu\text{m}$  cut was chosen mainly as a matter of practicality, as a smaller cut would have resulted in many more serial sections to be analyzed. Depending on the goals of future studies, a thinner cut might be recommended, but for our purposes the 10  $\mu\text{m}$  cut was adequate.

### *In vitro* culture of preantral follicles

One of the best tools available to give insight into early folliculogenesis is *in vitro* culture of preantral follicles. Although *in vitro* culture of preantral follicles



has been moderately successful in some species, attempts with large domestic farm animals have, for the most part, been less productive. The most successful culture systems have been for mice, where primordial follicles cultured *in vitro* then subsequently fertilized have produced embryos that were transferred into recipients that gave live birth (Eppig and O'Brien, 1996; O'Brien *et al.*, 2003; Wang *et al.*, 2011). Cultured preantral follicles have resulted in the production of viable embryos in several other species, including rats (Daniel *et al.*, 1989), pigs (Wu *et al.*, 2001), buffalo (Gupta *et al.*, 2008), sheep (Arunakumari *et al.*, 2010), and goats (Magalhães *et al.*, 2011).

Systems for oocyte culture have been established in mares (for review see Hinrichs, 2010), but only two studies exist on *in vitro* culture of equine preantral follicles. In an abstract by Szlachta and Tischner (2000), enzymatically isolated follicles cultured *in vitro* responded to FSH supplementation by experiencing an increase in growth rate after 1 day of culture followed by an increased rate of atresia thereafter until day 4 of culture. Szlachta and Tischner (2004) tested the efficacy of two different culture media (Menezo B2 and Wymouth MB 752/1) with and without the supplementation of FSH on the *in vitro* culture of preantral follicles isolated enzymatically. The results showed that Menezo B2 was the superior culture medium, while supplementation with FSH neither affected follicle growth rate nor increased follicle survival rates during the 4 day culture period. While these studies do offer some insight into determining the framework of a successful *in vitro* culture system for preantral follicles in the equine species, several additional *in vitro* culture studies are necessary to establish a good system for studying early folliculogenesis in mares.

Our recent work (Haag *et al.*, 2013b) is only the third known report on *in vitro* culture and the first known report on *in situ* culture of equine preantral follicles. The data generated from this preliminary study indicated that culturing preantral follicles *in situ* for 7 days in  $\alpha$ -MEM<sup>+</sup> promoted follicle development and follicle and oocyte growth, with 27% of the follicles maintaining morphological normality throughout the culture period. Several advantages related to *in situ* culture have been outlined, as reviewed by Picton *et al.* (2008) as follows: 1) damage caused to the follicle during isolation is avoided, 2) the chances of necrosis are minimized as the surface area of the tissue is maximized for gaseous exchange and fulfillment of nutrient requirements, and 3) a complex support system is provided that closely resembles the ovarian environment *in vivo*, keeping the follicles intact and in contact with the surrounding stromal cells which trigger the initiation of follicle growth under the control of local biochemical pathways. The BPU method is a feasible way to harvest ovarian fragments with a high proportion of primordial follicles

that can be directly submitted to culture without any additional manipulation or preparation, making it less time consuming than individually isolating follicles before culture, which could take several hours (Figueiredo *et al.*, 2011). These advantages have made *in situ* culture a successful means of primordial follicle activation and growth in several species, including mice (Eppig and O'Brien, 1996; O'Brien *et al.*, 2003), hamsters (Yu and Roy, 1999), baboons (Fortune *et al.*, 1998), humans (Hovatta *et al.*, 1999; Wright *et al.*, 1999; Telfer *et al.*, 2008), goats (Silva *et al.*, 2004a, b; Martins *et al.*, 2008), and cows (Wandji *et al.*, 1996; Fortune *et al.*, 1998; Gigli *et al.*, 2006; McLaughlin and Telfer, 2010). However, the surrounding cortical tissue might act as a barrier to medium perfusion during *in situ* culture, resulting in the development of primordial follicles only to the secondary stage (Martins *et al.*, 2008). Once the secondary stage is reached, those follicles can be isolated mechanically and further cultured individually to the antral stage (Eppig and O'Brien 1996; O'Brien *et al.*, 2003; McLaughlin and Telfer, 2010).

The superiority of  $\alpha$ -MEM<sup>+</sup> as a base culture medium to TCM-199<sup>+</sup> was indicated in our study (Haag *et al.*, 2013b) as  $\alpha$ -MEM<sup>+</sup> had a higher proportion of morphologically normal follicles after 1 day of culture and was the only medium in which follicles were found after 7 days of culture. The rich formulation of  $\alpha$ -MEM has been acknowledged and it has been used as a base medium for successful *in vitro* culture systems for preantral follicles in several species, including humans (Wright *et al.*, 1999), mice (Mousset-Simeón *et al.*, 2005), buffalo (Gupta *et al.*, 2008), dogs (Serafim *et al.*, 2010), and goats (Magalhães *et al.*, 2011). The additional supplementation of glutamine, hypoxanthine, BSA, ITS, and ascorbic acid has been shown to be essential for the survival of caprine preantral follicles during *in vitro* culture (Silva *et al.*, 2004a). In future studies, the effects of various hormones and growth factors need to be tested on equine preantral follicles in order to determine the optimal environment for mediating complete folliculogenesis *in vitro*.

## Conclusions

Our recent studies on equine preantral follicles (Haag *et al.*, 2013a, b, c) produced the following general findings: 1) the BPU method provided sufficient material for the study of early folliculogenesis in mares; 2) preantral follicle quantity, morphology, and viability did not differ according to phase of the estrous cycle; 3) number of follicles, but not follicle morphology or viability, was greater for younger versus older mares; 4) rate of atresia generally increased with follicle class; 5) proportion of primordial to primary follicles was higher for histological versus tissue chopper analysis, even though overall follicle morphology rates were similar;



6) rate of morphologically normal follicles, follicle viability, and follicle class proportions were similar between follicles from *in vitro* BPU and scalpel blade dissected fragments; 7) number of follicles per mg of tissue within animals was similar between tissue chopper and histological analyses; 8) preantral follicles submitted to an *in vitro* culture system appeared to respond positively by undergoing activation and growth during a 7 day culture period, with some follicles remaining morphologically normal; and 9)  $\alpha$ -MEM<sup>+</sup> seemed to be superior to TCM-199<sup>+</sup> as a base culture medium for equine preantral follicles.

In conclusion, the results from our recent studies validated the transvaginal, ultrasound-guided BPU method as a way to harvest ovarian fragments containing large numbers of morphologically normal and viable preantral follicles for the study of early folliculogenesis in the equine species. The ovarian fragments can be submitted to histology or processed using a tissue chopper for further analysis of the preantral follicles. The fragments can also be submitted directly to *in vitro* culture, where follicle growth and development can be achieved after 7 days of culture in  $\alpha$ -MEM<sup>+</sup> medium. Finally, the BPU method can be used to repeatedly harvest large numbers of primordial and primary follicles from equine ovaries without jeopardizing short-term reproductive function. Successful *in vitro* culture and cryopreservation systems for this species might make the BPU method a feasible technique to provide material to enable the utilization of oocytes within the abundant preantral follicles present in the equine ovary, many of which are essentially wasted *in vivo* through inactivation or atresia during development. In the future, these technologies could potentially provide a means for the preservation of genetic material from valuable individuals or for large-scale embryo production.

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## Metabolic hormones and reproductive function in cattle

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### Abstract

Diets can alter the concentrations of circulating hormones such as insulin and IGF-I. Such responsive hormones are related directly to nutritional status and moreover, directly or indirectly, associated with reproductive function and fertility. Metabolic hormones are involved in follicular development, number and size of ovarian structures, circulating concentrations of steroid hormones, duration of estrus, steroidogenesis, ovulation and embryonic development. However, circulating metabolic hormones in excess, resulting from high dry matter/energy intake can also contribute to the reduction of oocyte and embryo quality. Although changes in dietary intake affect ovarian function in *Bos taurus* and *Bos indicus* cattle, it seems that overfeeding influences more profoundly oocytes/embryos from heifers and cows of *Bos taurus* than of *Bos indicus* breeds. There is also a distinct effect of nutrition on *in vitro* vs. *in vivo* embryo production, in which metabolic hormones seem to affect more the later stages of follicle development. Thus, this paper presents and discusses the results of some relevant studies on the role of feed intake and its association with metabolic hormones in bovine reproduction.

**Keywords:** bovine, embryo, IGF-I, insulin, ovary, physiology.

### Introduction

Metabolic hormones are associated with diets provided to animals and have an important role in the regulation of reproductive activity from oogenesis or spermatogenesis, embryo development, and fetal growth until parturition. These hormones, mainly insulin and IGF-I are associated with aspects of physiology and reproductive performance in ruminants. Due to confounding factors related to hormones or other substances that change due to dietary alterations, it is often difficult to discern which factor is responsible for timely responses under different moments or feeding amounts.

This article aims to discuss findings from key studies that evaluated the influence of these metabolic hormones in ruminants, especially in the cow. In addition, we will present studies performed in our laboratory, where we observed the association of

metabolic hormones and reproductive aspects in cattle, often comparing animals of different genetic groups.

### Metabolic hormones

#### *Growth Hormone (GH)*

Growth hormone plays an important role in the regulation of ovarian function, however the exact mechanisms of its action are not well understood. It is known that nutritional deficiency leads to increased plasma GH, and its main effect appears to be in regulating the synthesis and release of IGF-I in the liver. However, there is a possibility that GH has a direct effect on the ovary due to the presence of mRNA for GH receptors (Gong *et al.*, 1991).

Chase *et al.* (1998) and Bossis *et al.* (1999) have shown that GH release is controlled by nutrition and IGF-I concentration by a negative feedback. However, the GH action on the IGF-I production is dependent on insulin. Underfed animals show high circulating GH and low insulin and IGF-I concentrations. This is due to a lower concentration of liver GH receptors (rGH), which may be due to lower induction of rGH and/or reduction in second-messenger signaling. Insulin induces active rGH and its absence leads to decreased responsiveness to GH (Chase *et al.*, 1998).

Butler *et al.* (2003) showed that insulin restores responsiveness to GH in dairy cows with negative energy balance (NEB) induced by lactation, by affecting the expression of IGF-I and rGH. IGF-I continuously increased during the infusion of insulin. Furthermore, higher expression of mRNAs for liver GH and IGF-I receptors were detected in cows infused with insulin compared to the control group. It was concluded that hypoinsulinemia in the postpartum period of dairy cows is responsible for the lower liver rGH expression, resulting in no GH binding and no subsequent secretion of IGF-I.

#### *IGF system*

Insulin-like growth factors (IGFs) are produced and secreted primarily by the liver in response to GH stimulus and may or may not be associated with nutrition but mainly due to insulin concentrations. The GHr is found in numerous tissues but it is plentiful in the

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liver. IGFs act as mediators of most growth-promoting actions of GH and are single-chain polypeptides with structural homology to proinsulin. IGFs regulate the proliferation and differentiation of many cell types and have insulin-like metabolic effects.

The IGF system is complex and consists of IGF-I and IGF-II binding to two types of receptors (type I and II), six IGF binding proteins, which are IGFBP-1, -2, -3, -4, -5 and -6, and an enzyme of inactivation (IGFBPase) of the IGFBPs. The low molecular weight (IGFBP-2, -4, and -5) binding proteins show greater affinities to IGF, which prevent IGFs to bind to their receptors. The IGFBPases degrade IGFBPs providing greater bioavailability of IGF (free) to bind to the receptors (Fortune *et al.*, 2004). The IGF-I stimulates cell proliferation and differentiation and acts synergistically with FSH on steroidogenesis by increasing the activity of P450 aromatase (Echternkamp *et al.*, 1994).

Webb *et al.* (2004) indicated that these factors play an important role in the initial stages of follicle development acting on granulosa cells of preantral follicles such that mRNA of both IGFBP-2 and IGF type I receptor are expressed. Thus, any change in the components of the IGF system can potentially affect follicular development. Moreover, several authors (Ginther *et al.*, 2001; Fortune *et al.*, 2004) showed that there is a strong involvement of the IGF system in the selection of the dominant follicle and changes in the IGF system are critical to the establishment of follicular dominance.

### Insulin

Insulin is secreted by pancreatic  $\beta$  cells and plays a central role in body metabolism. Besides its anabolic action, it also acts as a marker of energy status to the central nervous system. Besides acting as a potent stimulator of mitosis, physiological concentrations of insulin are probably necessary for normal follicle steroidogenesis in concert with the action of FSH (Bossis *et al.*, 1999; Buttler *et al.*, 2004). Insulin concentrations of approximately 100 ng/ml stimulate mRNA expression, P450 aromatase activity, and increase the secretion of estradiol (E2; Silva and Price, 2002).

Insulin acts as a signal to mediate the effects of acute changes in the diet on follicle dynamics in cattle. The reduction in fertility of dairy cows under NEB postpartum was associated with decreases in IGF-I and insulin. Insulin concentrations vary throughout the day as well as during the estrous cycle, with a significant increase during the preovulatory period. Estradiol is a strong candidate for mediating these changes, because the increase of insulin occurs in parallel to the increase of E2 associated with the development of the dominant follicle. Estradiol has been shown to stimulate the expression of both insulin mRNA and insulin secretion by the pancreas (Webb *et al.*, 2004). We observed

(Bastos *et al.*, 2010) that pre-prandial insulin concentrations were higher in the estrogenic than the progestational phase of the estrous cycle in both Nelore ( $11.9 \pm 2.05$  vs.  $8.3 \pm 1.47$   $\mu$ U/ml) and Holstein ( $4.2 \pm 2.2$  vs.  $1.05 \pm 0.64$   $\mu$ U/ml) cows.

### Glucose

Glucose appears to be a metabolic signal generating information for controlling GnRH secretion. According to its bioavailability, glucose acts within the central nervous system for detecting peripheral glucose status to modulate GnRH and, indirectly, LH secretion. Glucose bioavailability influences both tonic and preovulatory centers to regulate GnRH release and thus LH secretion. Animals with hypoglycemia had a delayed LH release and glucose infusion restored the normal time of LH release induced by E2 (Diskin *et al.*, 2003).

### Nutrition and metabolic hormones in *Bos taurus* vs. *Bos indicus*

With the purpose of comparing nutritional and metabolic aspects between *Bos taurus* and *Bos indicus*, a study was conducted with non-lactating Nelore ( $n = 7$ ) vs. Holstein ( $n = 8$ ) cows to evaluate the profile of feed consumption and circulating concentrations of glucose and insulin pre- and post-prandially (Ishiguro and Sartori, 2013; ESALQ/USP, Piracicaba; unpublished observations). The diet was calculated for maintenance by the National Research Council - NRC (2001). Holstein cows spent more time ( $135.0 \pm 7.0$  min) ingesting food than Nelore cows ( $104.4 \pm 5.9$  min). There were differences in circulating glucose and insulin which were higher ( $P < 0.05$ ) in Nelore than Holstein cows, as shown in Fig. 1. The substantial difference in circulating insulin, but a discrete difference in glucose between Nelore and Holstein cows are very interesting observations and suggest distinct mechanisms for control of insulin increase after feeding as well as for regulation of glucose turnover between the two breeds.

### Influence of nutrition / metabolic hormones on the number of antral follicles

The nutritional flushing prior to ovarian superstimulation may increase follicular population and superovulatory response in cows, which may be associated with increased insulin and IGF-I concentrations in response to higher propionate concentrations. Studies with beef heifers showed that overfeeding for a short period (up to 3 weeks) increased circulating insulin and the number of follicles (Gutierrez *et al.*, 1997), improving superovulatory response (Gong *et al.*, 2002). However, other studies found no effect or described a negative relationship between feed intake



and follicular population, including those developed in our laboratory when crossbred cows (Bastos *et al.*, 2009) or *Bos indicus* heifers (Mollo *et al.*, 2007a) were used. Due to the fact that BCS at the beginning of the nutritional flushing may influence embryo production in *Bos taurus* cattle (Adamiak *et al.*, 2005), we designed a study to investigate whether differences in the BCS were associated with the nutritional flushing influence to the superovulatory response in Nelore heifers (Bastos *et al.*, 2007). Thirty-six pubertal heifers with lower ( $2.7 \pm 0.1$ ,  $n = 18$ ) or higher ( $3.7 \pm 0.1$ ,  $n = 18$ ) BCS (scale 1-5) were divided into two groups which were subdivided according to the nutritional requirements in maintenance (M) or Flushing (1.8M). This design resulted in four subgroups: <BCS + Maintenance (<M); <BCS + Flushing (<F); >BCS + Maintenance (>M) and >BCS + Flushing (>F). The nutritional flushing was done during 14 days before the first FSH injection for superovulation, when heifers returned to the maintenance diet. Each heifer was superovulated twice and the interval between procedures was 35 days. The number of follicles  $\geq 3$  mm at the time of the first FSH injection did not differ among groups >M, >F, <M, <F, respectively ( $56.4 \pm 6.0$ ,  $55.1 \pm 4.5$ ,  $54.3 \pm 6.9$  and  $48.2 \pm 4.8$ ;  $P > 0.10$ ). There was also no difference in superovulatory response among groups (discussed below).

One of the studies showing negative relationship between nutritional intake and follicular population used 39 *Bos indicus* heifers that were fed

with 40.8% of coast-cross hay, 51.9% corn silage and 7.3% mix (energy, urea, minerals, and vitamins) for 9 weeks (Mollo *et al.*, 2007a). The heifers were divided into two groups according to the dietary levels based on the maintenance: 1.7 M and 0.7 M. At the end of the seventh week, heifers were subjected to superovulatory treatments. Despite the fact that the heifers in group 1.7 M had higher BCS, higher body weight and more blood insulin concentrations ( $14.3 \pm 1.7$  vs.  $3.0 \pm 0.8$   $\mu\text{IU/ml}$ ), they had lower number of follicles  $\geq 3$  mm at the first FSH treatment ( $32.6 \pm 2.5$  vs.  $42.6 \pm 6.6$ ;  $P = 0.10$ ). Superovulatory data of this study will be ahead.

A stimulatory effect of bovine somatotropin (bST) on follicle population has been described in *Bos taurus* and *Bos indicus* cattle. Treatment with recombinant bST increased the number of follicles (6 to 15 mm) in lactating Holstein cows and size of second largest ovarian follicles in both lactating and non-lactating cows (De la Sota *et al.*, 1993). Moreover, bST-treated lactating dairy cows before day 12 (first follicular wave, estrus = day 0), had more ovarian follicles between 3 and 9 mm than saline-treated cows (Lucy *et al.*, 1993). Also, Buratini *et al.* (2000) observed a significant increase in plasma IGF-I concentration and a 36% increase in number of small follicles (<5 mm) when Nelore heifers ( $n = 8$ ) were treated with bST on day 3 of the estrous cycle. However, there was no effect on the number of medium (5-9 mm) or large (>9 mm) follicles.

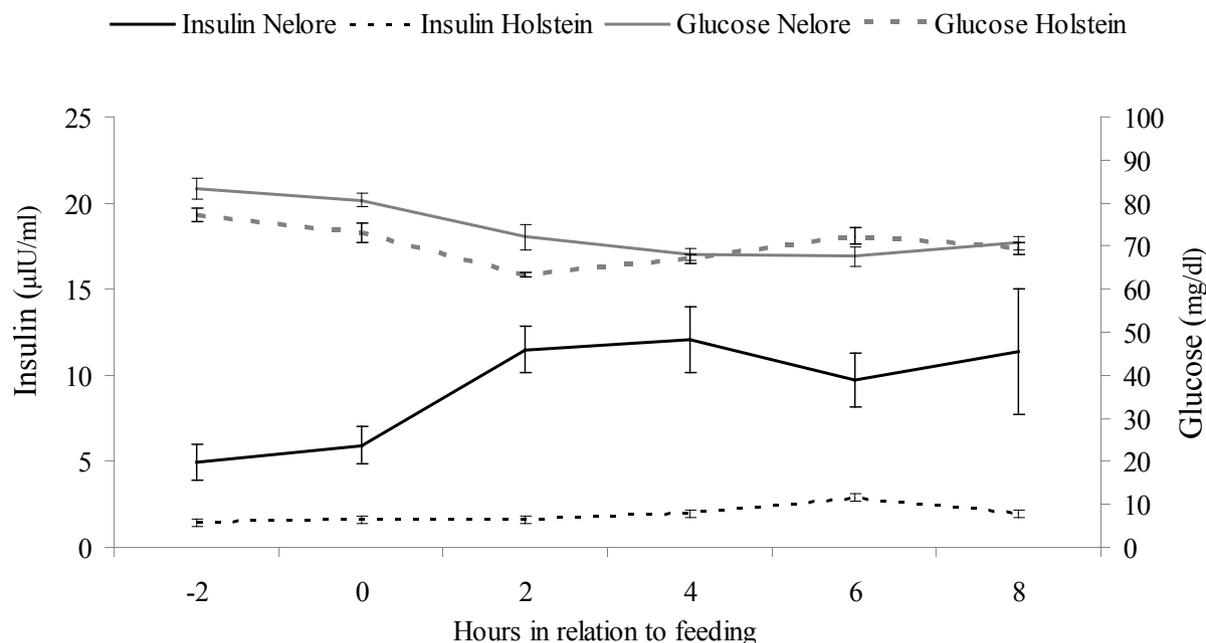


Figure 1. Circulating concentrations of glucose and insulin in Nelore ( $n = 7$ ) vs. Holstein cows ( $n = 8$ ) pre- and post-prandially. Breed; day; breed x day ( $P < 0.05$ ) for glucose and insulin.



### Influence of feed intake on ovarian structures and steroid hormones

High feed intake can alter ovarian physiology and hormonal concentrations. In lactating dairy cows there is a high positive correlation ( $r = 0.88$ ) between DMI and milk production (Harrison *et al.*, 1990). Even though high producing dairy cows have larger follicles, serum E2 concentrations were lower when compared to heifers and dry cows (De la Sota *et al.*, 1993; Sartori *et al.*, 2002; Wolfenson *et al.*, 2004). The reasons for lower circulating steroid hormone concentrations in cows with higher DMI are probably related to an increased metabolism of these hormones as discussed by Wiltbank *et al.* (2006).

We compared the ovarian function of pubertal heifers ( $n = 27$ ) and lactating dairy cows producing  $45.7 \pm 1.3$  kg/d of milk ( $n = 14$ ) during an estrous cycle (Sartori *et al.*, 2004). Likewise, we followed the estrous cycle of pubertal heifers under high (1.7 M) or low (0.7 M) feed intake (Mollo *et al.*, 2007b). In these and other studies, there was an association of high DMI with larger sizes of ovarian structures, and lower circulating steroid hormones concentrations. For example, in a recent study (Bastos and Sartori, 2013; ESALQ/USP, Piracicaba; unpublished data), non-lactating Nelore and Holstein cows, were exposed to high or low feed intake. Regardless of the genetic group, overfed cows had higher CL volume ( $5,146 \pm 287$  vs.  $3,964 \pm 306$  mm<sup>3</sup>;  $P < 0.01$ ), and lower plasma P4 concentrations ( $2.3 \pm 0.2$  vs.  $3.0 \pm 0.2$  ng/ml;  $P < 0.03$ ) than those with low feed intake. Likewise, the cows exposed to high DMI had larger ovulatory follicles ( $15.1 \pm 0.8$  vs.  $13.8 \pm 0.8$  mm;  $P < 0.01$ ) and lower plasma E2 preovulatory peak ( $12.6 \pm 1.0$  vs.  $16.0 \pm 1.0$  pg/ml;  $P < 0.01$ ). Interestingly, regardless of genetic group, overfed cows had follicle deviation occurring later in the cycle ( $2.9 \pm 0.2$  vs.  $2.5 \pm 0.2$  days after ovulation;  $P < 0.05$ ) and the diameter when the largest follicle reached deviation was also greater ( $8.1 \pm 0.2$  vs.  $7.4 \pm 0.2$  mm;  $P < 0.05$ ).

### Influence of dry matter or energy intake associated with high insulin on yield and quality of embryos

Most researchers who have studied the effect of feed intake on embryo production reported negative results on the reproductive function of cattle overfed compared with those fed restricted diets (see details in Sartori *et al.*, 2012). The causes of impaired embryo production related to feed intake are still not well understood. However, changes in liver blood flow, in local and circulating metabolites (glucose and IGF-I), in hormone concentrations (insulin and steroids), and in different sources of volatile fatty acids may be involved in these processes. Moreover, we hypothesized that the effects of dietary intake on embryo quality may differ between *Bos taurus* and *Bos indicus* cattle, which

consistently show different concentrations of circulating insulin and IGF-I concentrations (Sartori *et al.*, 2010). As shown below, it is tempting to speculate that Zebu cattle may be more resistant than European breeds to the effects of changes in feed intake on embryo production and quality.

Changes in DMI as discussed above, may affect the blood concentrations of steroid hormones, IGF-I and insulin, and affect oocyte quality, fertilization or embryo/oocyte transport and early embryonic development (Folman *et al.*, 1973, Fonseca *et al.*, 1983; Mann *et al.*, 1998; Inskeep, 2004), resulting in reduced fertility. In addition to the effects of IGF-I and insulin in steroidogenesis (Gutierrez *et al.*, 1997; Armstrong *et al.*, 2002; Gong *et al.*, 2002) and the sensitivity of the follicle to gonadotropic hormones (Webb *et al.*, 2004), as already described, hyperinsulinemia and increased plasma and intrafollicular IGF-I concentrations impair oocyte quality and subsequent embryo development of *Bos taurus* cattle (Armstrong *et al.*, 2001; Adamiak *et al.*, 2005). In our studies, we investigated how the DM or energy affects *in vivo* and *in vitro* embryo production in Nelore cattle.

In the study by Mollo *et al.* (2007a), after feeding treatment diets for 9 weeks, overfed heifers (1.7 M,  $n = 20$ ) presented lower superstimulatory ( $24.0 \pm 1.1$  vs.  $48.4 \pm 1.6$  follicles  $\geq 6$  mm;  $P < 0.001$ ) and superovulatory ( $15.7 \pm 0.9$  vs.  $33.6 \pm 1.4$  corpora lutea;  $P < 0.0001$ ) responses in comparison to those in restricted diet (0.7 M,  $n = 19$ ). Moreover, the number of recovered embryos/ova ( $6.7 \pm 0.9$  vs.  $10.5 \pm 0.6$ ;  $P < 0.0003$ ) and transferable embryos ( $3.8 \pm 0.4$  vs.  $5.7 \pm 0.6$ ;  $P < 0.01$ ) were also lower for the high feed intake heifers. The superstimulatory and superovulatory responses and the number of total and transferable embryos seemed to be compromised by higher circulating insulin concentrations at the first day of FSH treatment ( $14.3 \pm 1.7$  vs.  $3.0 \pm 0.8$   $\mu$ IU/ml;  $P < 0.001$ ). Regardless of treatment, there was a negative correlation ( $-0.61$ ;  $P < 0.05$ ) between circulating insulin concentration and the difference in the number of follicles in the ovaries between the last and first day of FSH treatment.

Bastos *et al.* (2007) did not detect any effect on the superovulatory response, embryo production, or embryo quality in heifers ( $n = 36$ ) with higher or lower BCS fed maintenance (M) or flushing (1.8 M) diets for 14 days before a superovulation treatment. In the study of Surjus *et al.* (2012), little variation of the superstimulatory response ( $14.6 \pm 1.6^a$  vs.  $12.6 \pm 1.4^b$  vs.  $13.6 \pm 1.5^{ab}$  number of follicles  $>6$  mm;  $P < 0.05$ ) was reported in non-lactating cows ( $n = 32$ ) fed maintenance (M, 1.2% of DM/kg of BW), 0.7 M (0.84% of DM/kg of BW) or 1.5 M (1.8% of DM/kg of BW) diets after 42 days of feeding, in a latin-square design. There was no difference for the superovulatory response ( $11.0 \pm 1.4$  vs.  $9.8 \pm 1.3$  vs.  $10.2 \pm 1.3$  corpora lutea;  $P > 0.10$ ), fertilization rate ( $P = 0.71$ ) or percentage of viable embryos ( $P = 0.98$ ) among



experimental groups (Surjus *et al.*, 2012). Regardless of treatment, circulating insulin at the beginning of superovulation, was negatively correlated with superovulatory response ( $r = -0.32$ ) and number of viable embryos ( $r = -0.22$ ). Pregnancy rates at 23 and 53 days after embryo transfer did not differ between treatments; however, the circulating concentrations of insulin in donors had a low, but significant negative correlation with pregnancy of recipients at 60 days of gestation ( $r = -0.16$ ;  $P < 0.05$ ).

Guardieiro *et al.* (2013) supplied concentrate with or without rumen-protected fat (Megalac-E, rich in linoleic acid) to 40 heifers starting 50 days before superovulation, in a cross-over experimental design. Supplemental diets were isocaloric and isonitrogenous. The embryos recovered were cryopreserved and subsequently evaluated for *in vitro* embryo development. The superstimulatory response, number of total embryos/ova, viable embryos, degenerate embryos, or unfertilized oocytes recovered were similar between groups. However, there was negative effect of unsaturated fatty acids on the superovulatory response ( $15.7 \pm 1.2$  vs.  $18.0 \pm 1.3$  corpora lutea;  $P = 0.06$ ), hatching rate at 48 hours ( $17.3 \pm 3.3\%$ ;  $n = 137$  vs.  $33.1 \pm 4.0\%$ ,  $n = 148$ ,  $P < 0.009$ ) and at 72 h ( $30.9 \pm 4.0\%$ ,  $n = 137$  vs.  $44.3 \pm 4.2\%$ ;  $n = 148$ ;  $P < 0.04$ ) of *in vitro* culture. This negative effect associated with a rumen bypass fat diet may have been influenced by lower plasma concentrations of IGF-I observed in this experimental group compared to control ( $374.3 \pm 27.2$  vs.  $483.8 \pm 26.5$  ng/ml, respectively;  $P < 0.0001$ ).

Our group also performed two studies to investigate the effects of feed intake on *in vitro* embryo production (Martins *et al.*, 2006; Prata *et al.*, 2011). At the first study, overfed cows (1.7 M,  $n = 10$ ) in comparison to those underfed (0.7 M,  $n = 10$ ) had a small but significant increase in number of follicles  $\geq 3$  mm in diameter at the time of ovum pick-up (OPU) and associated with this effect was a tendency for a greater circulating concentration of insulin in the 1.7 M group ( $5.6 \pm 0.8$  vs.  $3.5 \pm 0.7$   $\mu$ IU/ml;  $P = 0.06$ , unpublished). Moreover, the diet with higher energy content tended to reduce the percentage of viable oocytes ( $44.0\%$ ,  $n = 732$  vs.  $48.6\%$ ,  $n = 623$ ;  $P = 0.08$ ). In despite of similar numbers of cleaved oocytes and blastocysts between groups, there was a higher expression of the BAX gene and global expression of all evaluated genes on embryos from the lower feed intake group, which may be related to better embryo quality (Martins *et al.*, 2006). A study by Prata *et al.* (2011) used the same design as described by Surjus *et al.* (2012), however cows underwent OPU 30 days after the dietary treatments had started. More recovered oocytes ( $20.2 \pm 2.0^b$ ,  $23.0 \pm 2.3^a$ , and  $21.5 \pm 2.2^{ab}$ ;  $P < 0.02$ ) and viable oocytes ( $14.4 \pm 1.6^b$ ,  $17.0 \pm 1.9^a$ , and  $15.7 \pm 1.7^{ab}$ ;  $P < 0.006$ ) were detected in the 0.7 M diet in relation to the M diet. Surprisingly, cows receiving the 1.5 M diet did not differ from the other groups.

Although the number of cleaved oocytes was also higher in 0.7 M cows as compared to M cows ( $10.7 \pm 1.4^b$ ,  $13.4 \pm 1.7^a$ , and  $12.6 \pm 1.6^{ab}$  for M, 0.7 M, and 1.5 M;  $P < 0.04$ ), we did not detect influence of diet on the number ( $5.4 \pm 0.8$ ,  $6.9 \pm 0.9$ , and  $5.9 \pm 0.8$ ;  $P = 0.15$ ) or percentage of blastocysts produced *in vitro* (31.9, 30.6, and 31.1%;  $P = 0.67$ ). Moreover, regardless of treatment, cows with lower plasma insulin concentration ( $3.1 \pm 0.75$   $\mu$ IU/ml) showed similar results ( $P > 0.10$ ) as compared to cows with higher circulating insulin ( $9.72 \pm 0.21$   $\mu$ IU/ml) in all of the variables analyzed, such as blastocyst rate (24.6 vs. 25.9%) and conception rate [31.2% (69/221) vs. 33.3% (71/213)] at 30 days.

## Conclusions

Metabolic hormones are key elements to the reproductive performance, once they can affect different aspects of the physiology of the cow. The IGF system, as well as insulin, associated with reproductive hormones, interacts with ovarian activity, from the initial stages of follicular growth, to the process of selection of the dominant follicle, as well as final development and ovulation. When considering the effects of high DMI / energy on bovine fertility, the negative effects are highlighted, because they are associated with a high metabolism of steroid hormones and with an increase in circulating insulin and IGF-I concentrations, potentially compromising oocyte / embryo quality and lowering conception rates. Interestingly, although controversial, negative effects of hyperinsulinemia seem to be more pronounced in embryos produced *in vivo* than *in vitro*. Thus, we may speculate that in small follicles ( $< 7$  mm), this impairment on oocyte quality caused by higher circulating insulin and IGF-I concentrations is less intense, especially in *Bos indicus* cattle. In contrast, low circulating IGF-I may compromise follicle number and development as well as embryo production and cryotolerance.

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## Relationships between growth of the preovulatory follicle and gestation success in lactating dairy cows

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### Abstract

This report summarizes three studies conducted with lactating dairy cows aiming to increase pregnancy rates to fixed time artificial insemination (TAI) protocols. Experiment 1 was designed to determine if changing the timing of PGF2 $\alpha$  treatment during an E2/P4-based program would affect fertility to TAI or fixed-time embryo transfer (TET). In experiment 2, pregnancy rates to AI were compared following synchronized ovulation using two protocols that have been developed to reduce the period between follicular wave emergence and TAI. The Ovsynch-type protocol utilizes GnRH to synchronize the follicular wave by inducing ovulation of a dominant follicle at the beginning of the protocol, and to synchronize ovulation at the end of the protocol allowing TAI. In contrast, E2/P4-based protocols utilize E2 products in the presence of P4 to induce atresia of antral follicles and synchronize emergence of a new follicular wave. At the end of E2/P4-based protocol another E2 treatment in the absence of P4 is used to induce LH release and synchronize ovulation and allow TAI. Experiment 3 was designed to determine whether increasing the length time interval with reduced circulating P4 (proestrus) would increase fertility in a TAI program that utilized E2 and P4 to synchronize ovulation of cycling, lactating dairy cows. The overall conclusions are that circulating concentrations of progesterone and estradiol prior to and circulating concentrations of progesterone following ovulation can affect fertility in cattle. In addition, small increases in P4 concentrations near the time of AI, due to lack of complete CL regression, result in reductions in fertility. Earlier treatment with PGF2 $\alpha$  should allow greater time for CL regression, an increase in estradiol and subsequent reductions in circulating P4 that could be critical for fertility. Optimization of follicle size in TAI programs is clearly an intricate balance between oocyte quality, adequate circulating E2 near AI, and adequate circulating P4 after AI.

**Keywords:** fertility, proestrus lactating dairy cows.

### Introduction

Hormonal treatments have been developed to synchronize the time of ovulation in dairy cattle, allowing successful fixed time artificial insemination (TAI) without the need for detection of estrus (Pursley *et al.*, 1997).

In dairy cows a variety of methods have been evaluated to increase fertility during synchronization of ovulation programs including: increasing progesterone (P4) concentration during ovulatory follicle development (Bisinotto *et al.*, 2010; Martins *et al.*, 2011; Wiltbank *et al.*, 2011), increasing length of proestrus (Peters and Pursley, 2003; Pereira *et al.*, 2013), reducing follicle age (Cerri *et al.*, 2009; Santos *et al.*, 2010), supplementing estrogen (E2) during proestrus (Cerri *et al.*, 2004; Brusveen *et al.*, 2009; Souza *et al.*, 2011) or increasing P4 after AI (Demetrio *et al.*, 2007; Lonergan, 2011).

This report summarizes three studies conducted with lactating dairy cows aiming to increase pregnancy rates to TAI protocols.

### Materials and Methods and Results

#### Experiments

Experiment 1 (Pereira *et al.*, 2013) was designed to determine if changing the timing of PGF2 $\alpha$  treatment during an E2/P4-based program would affect fertility to TAI or fixed-time embryo transfer (TET). The experiment was conducted on a total of 1,058 lactating Holstein cows at eleven commercial dairy farms in Paraná State, Brazil. Within each farm, cows were randomly assigned to receive one of the following treatments for synchronization of ovulation: 1) an intravaginal P4 device containing 1.9 g of P4 (CIDR<sup>®</sup>, Zoetis, São Paulo, Brazil) and 2.0 mg (i.m.) estradiol benzoate (Estrogin<sup>®</sup> - Farmavet, São Paulo, Brazil) on day 0, 25 mg (i.m.) dinoprost tromethamine (Lutalyse<sup>®</sup>, Zoetis) on day 7 or 8, CIDR removal and 1.0 mg (i.m.) of estradiol cypionate (ECP<sup>®</sup>, Zoetis) on day 8. On day 8, cows were randomly assigned to receive either TAI

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on day 10 (48 h after ECP;  $n = 406$ ) or TET on day 17 ( $n = 652$ ). All TET cows received 100  $\mu\text{g}$  (i.m.) of gonadorelin (Fertagyl, MSD Animal Health, São Paulo, Brazil) at time of ET (Vasconcelos *et al.*, 2011). In a subgroup of cows ( $n = 444$ ) in both groups, ovaries were evaluated by transrectal ultrasonography (Aloka SSD-500 with a 7.5-MHz linear-array transducer, Aloka, Tokyo, Japan) on day 10 to measure the diameter of the largest follicle present prior to ovulation. Milk production was measured daily between days 10 and 17, and average daily production through this interval was used in the analysis.

Table 1 shows the effect of treatment on pregnancies at days 28 and 60 (P/AI and P/ET). There was a clear effect of breeding technique (AI vs. ET) and treatment (PGF $_{2\alpha}$  on day 7 vs. day 8) on results of both days 28 and 60 of pregnancy. However, there was no significant interaction between breeding technique and treatment at either pregnancy diagnosis. Treatment with PGF $_{2\alpha}$  on day 7 increased fertility, compared to treatment on day 8, at day 28 of pregnancy. There was no effect of day of PGF $_{2\alpha}$  treatment on pregnancy loss, while pregnancy loss was higher following TET regardless of whether they received PGF $_{2\alpha}$  on day 7 or 8.

Table 1. Pregnancy per AI (TAI) or embryo transfer (TET) on days 28 and 60 and pregnancy losses for lactating dairy cows receiving PGF $_{2\alpha}$  treatment on days 7 or 8 during an E2/P4-based synchronized ovulation program.

Breeding Technique	Protocol	Pregnancy <sup>1</sup>		Pregnancy loss <sup>1</sup>
		day 28	day 60	
TAI	PGF $_{2\alpha}$ day 7	32.9 (87/238)	30.0 (81/238)	7.7 (6/87)
	PGF $_{2\alpha}$ day 8	20.6 (42/168)	19.2 (40/168)	5.5 (2/42)
TET	PGF $_{2\alpha}$ day 7	47.0 (116/243)	37.9 (95/243)	20.4 (21/116)
	PGF $_{2\alpha}$ day 8	40.7 (100/244)	33.5 (83/244)	19.4 (17/100)
<i>P</i> - values				
Breeding technique (AI vs. ET)		0.0006	0.021	0.012
Treatment (day 7 vs. day 8)		0.004	0.016	0.676
Breeding technique x Treatment		0.355	0.308	0.872

<sup>1</sup>Each value includes least-squares means % (no./no.).

There was no effect of treatment on the mean P4 concentration at the time of PGF $_{2\alpha}$  injection or on follicle size on day 10 (time of TAI). Figure 1 shows the differences between treatment groups in the distribution of P4 concentration on day 10. Treatment with PGF $_{2\alpha}$  on day

7 increased the percentage of cows with very low P4 concentrations on day 10 ( $\leq 0.09$  ng/ml). Delaying PGF $_{2\alpha}$  treatment until day 8 increased the percentage of cows with P4 concentrations between 0.1 and 0.21 ng/ml but had no effect on the percentage of cows with P4 > 0.21 ng/ml.

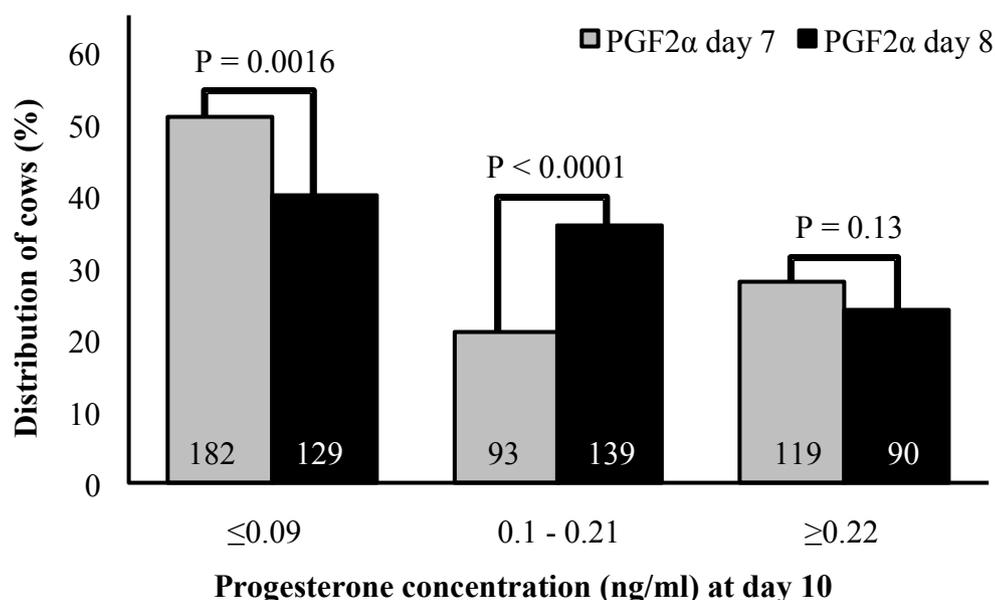


Figure 1. Effect of treatment with PGF $_{2\alpha}$  on day 7 ( $n = 394$ ) or day 8 ( $n = 358$ ) on the distribution of cows with different progesterone concentrations at day 10 (at AI or 7 days before ET).



The fertility of cows based on the 60 day pregnancy diagnosis was influenced by P4 concentration on day 10 as shown in Table 2. At the 28 day pregnancy diagnosis in cows that received TAI, the lowest P4 concentrations on day 10 ( $\leq 0.09$  ng/ml) resulted in the greatest P/AI (34.1%) with a 13.9% decrease in P/AI for cows with P4 levels of 0.10 to

0.21 ng/ml (68.8% relative difference) and a 12.7% decrease in P/AI for cows with P4 levels  $\geq 0.21$  ng/ml (59.4% relative decrease). In contrast, cows that received TET did not show a difference in P/ET when P4 levels of  $\leq 0.09$  ng/ml were compared to 0.1 to 0.21 ng/ml. However, there was a decrease of 20.9% in P/ET at day 60 in cows with P4 levels  $\geq 0.21$  ng/ml on day 10.

Table 2. Effect of progesterone concentrations on day 10 (at AI or 7 days before ET) on day 60 of pregnancy in lactating dairy cows after fixed timed artificial insemination (TAI) or timed embryo transfer (TET).

Item <sup>1</sup>	Progesterone (ng/ml) on day 10			P - value
	$\leq 0.09$	0.10 - 0.21	$\geq 0.22$	
TAI P/AI at day 60				
PGF2 $\alpha$ day 7	39.4 (36/85)	27.5 (8/26)	24.0 (12/45)	–
PGF2 $\alpha$ day 8	23.2 (15/54)	15.1 (8/45)	14.6 (4/22)	–
Combined <sup>2</sup>	34.1 (51/139) <sup>ax</sup>	20.2 (16/71) <sup>b</sup>	21.4 (16/67) <sup>by</sup>	0.05
TET P/ET at day 60				
PGF2 $\alpha$ day 7	46.8 (37/77)	44.2 (23/52)	25.3 (12/49)	–
PGF2 $\alpha$ day 8	40.0 (24/58)	46.0 (33/73)	20.5 (9/50)	–
Combined <sup>2</sup>	43.8 (61/135) <sup>a</sup>	45.3 (55/125) <sup>a</sup>	22.9 (21/99) <sup>b</sup>	0.0006

<sup>1</sup>Each value includes least-squares means % (no./no.). <sup>2</sup>Combined values of treatments to determine the effect of progesterone at day 10 on P/AI or P/ET. <sup>a,b</sup>Values with different superscripts in the same row differ ( $P < 0.05$ ). <sup>x,y</sup>Values with different superscripts in the same row tended to differ ( $P > 0.05$  and  $P \leq 0.1$ ).

In experiment 2 (Pereira *et al.*, Department of Animal Production, FMVZ-UNESP, Botucatu, SP, Brazil; unpublished data) pregnancy success to AI were compared following synchronized ovulation using two protocols that have been developed to reduce the period between follicular wave emergence and TAI. The Ovsynch-type protocol utilizes GnRH to synchronize the follicular wave by inducing ovulation of a dominant follicle at the beginning of the protocol, and to synchronize ovulation at the end of the protocol allowing TAI. In contrast, E2/P4-based protocol utilizes E2 products in the presence of P4 (in our study 2.0 mg EB) to induce atresia of antral follicles and synchronize emergence of a new follicular wave. At the end of E2/P4-based protocol another E2 treatment (in our study 1.0 mg ECP) in the absence of P4 is used to induce LH release and synchronize ovulation and allow TAI.

This study used a total of 1,190 lactating Holstein cows. Within each farm ( $n = 4$ ), cows were blocked by parity (primiparous and multiparous) before randomization. Within each block cows were randomly assigned to receive one of two treatments: 1) The 5-days Cosynch protocol consisting of an intravaginal progesterone device containing 1.9 g of P4 (CIDR), and 100  $\mu$ g i.m. of gonadorelin (Fertagyl), five days later the CIDR was removed and cows received PGF2 $\alpha$  i.m. (Lutalyse), a second i.m. PGF2 $\alpha$  was performed 24 h later, the final GnRH treatment was administered and TAI was performed 48 h after the second PGF2 $\alpha$  i.e. 72 h after CIDR removal; and 2) The E2/P4 protocol consisted of CIDR insertion and 2.0 mg i.m. of EB (Estrogin), 7 days

later 25 mg of i.m. PGF2 $\alpha$  (Lutalyse), 24 h later the CIDR was removed and cows received 1.0 mg i.m. of estradiol cypionate (ECP), and 48 h after CIDR removal TAI was performed. Cows were scanned on days -10, -3, and 0. Milk production was measured daily between days 0 and 7, and average daily production through this interval was used in the analysis. Cows were considered to have the estrous cycle synchronized when P4 was  $\geq 1.0$  ng/ml on day 7 and luteolysis was considered to have occurred when P4  $\leq 0.4$  ng/ml on day 0.

Table 3 shows the effect of treatments on luteolysis, estrus detection, estrous cycle synchronization, P/AI, and pregnancy loss between days 32 and 60. The proportion of cows that had luteolysis did not differ between treatments. The cows in the E2/P4 protocol were more likely to be detected in estrus compared with 5-days Cosynch protocol. A greater percentage of cows in the 5-days Cosynch protocol had their estrous cycles synchronized, compared with cows in the E2/P4 protocol. When all cows were included in the analysis, P/AI on day 32 was not affected by treatment, but there was a tendency ( $P = 0.07$ ) for cows in the E2/P4 protocol to have greater P/AI on day 60 after AI compared with cows in the 5-days Cosynch protocol. Percentage of cows that had pregnancy loss between days 32 and 60 after AI was lower in the E2/P4 program compared with the 5-days Cosynch program. In cows that had their estrous cycle synchronized, the E2/P4 protocol had greater P/AI on day 60 after AI and lower pregnancy loss between days 32 and 60 compared with cows in the 5-days Cosynch protocol.



Table 3. Luteolysis, estrus detection, estrous cycle synchronization, P/AI at days 32 and 60 and pregnancy loss for lactating dairy cows receiving 5-day Cosynch or E2/P4 protocol.

Item <sup>1</sup>	Treatment		P - value
	5-day Cosynch	E2/P4	
Luteolysis <sup>2</sup>	91.4 (389/424)	90.6 (380/418)	0.67
Estrus detection	43.4 (253/597)	62.8 (375/593)	<0.01
Estrous cycle synchronization <sup>3</sup>	78.2 (287/389)	70.7 (250/380)	0.02
P/AI			
At day 32	20.5 (119/597)	23.2 (135/593)	0.25
At day 60	16.7 (95/597)	20.7 (119/593)	0.07
Pregnancy loss	19.6 (24/119)	11.0 (16/135)	0.05
P/AI <sup>3</sup>			
At day 32	23.0 (66/287)	28.0 (70/250)	0.18
At day 60	17.7 (51/287)	25.6 (64/250)	0.03
Pregnancy loss	21.7 (15/66)	6.7 (6/70)	0.01

<sup>1</sup>Least squares means % (No./No.). <sup>2</sup>Cows that had luteolysis (P4  $\leq$ 0.4 ng/ml at day 0). <sup>3</sup>Cows that had luteolysis and their estrous cycle synchronized (P4  $\geq$ 1.0 ng/ml at day 7).

On the day of PGF injection, the P4 concentrations were greater for 5-days Cosynch protocol ( $2.7 \pm 0.13$  ng/ml) compared with E2/P4 protocol ( $1.7 \pm 0.13$  ng/ml). The follicle diameter at AI (day 0) had an effect on estrous cycle synchronization in both treatments, within larger follicles associated with greater estrous cycle synchronization. The data for P/AI by follicle diameter were evaluated only in cows that had their estrous cycle synchronized. In the 5-days

Cosynch protocol P/AI at 32 days and day 60 were linearly associated with the follicle diameter while in the E2/P4 program, the effect was curvilinear; there was a decreased P/AI at days 32 and 60 with very small and very large follicles (Fig. 2A and B). The follicle diameter affected pregnancy loss in the 5-days Cosynch protocol, with smaller follicles resulting in greater pregnancy loss and between days 32 and 60, but there was no effect in the E2/P4 protocol (Fig. 3).

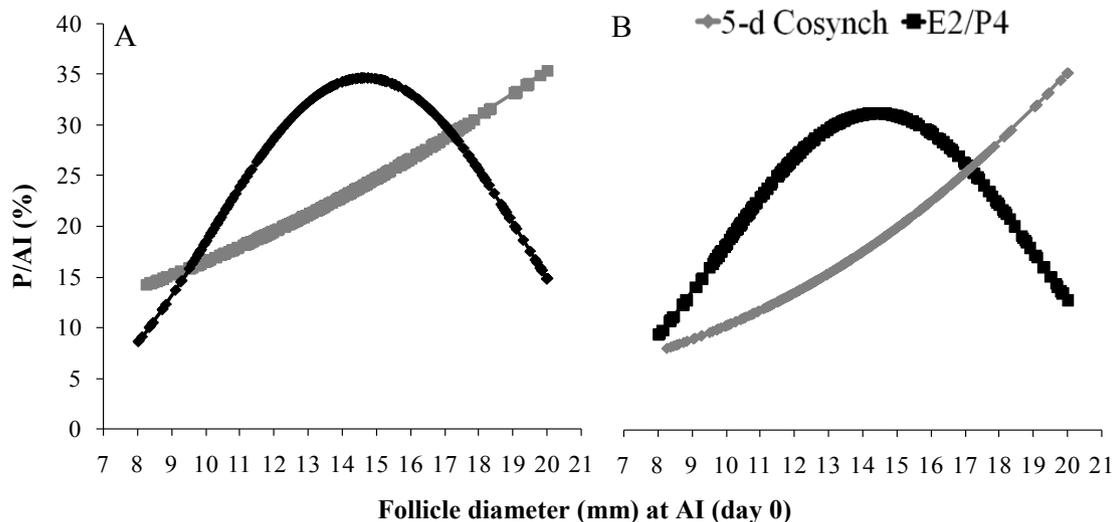


Figure 2. Effect of follicle diameter at AI (day 0) on P/AI at days 32 (Panel A) and 60 (Panel B) in cows that had their estrous cycle synchronized (P4  $\leq$ 0.4 ng/ml at day 0 and P4  $\geq$  1.0 ng/ml at day 7) receiving E2/P4 or 5-day Cosynch protocols.

Expression of estrus improved the percentage of cows that had their estrous cycle synchronized (no estrus = 63.8% [220/357], estrus = 83.4% [317/387]) independent of treatment. Expression of estrus was associated with increased P/AI at 32 days (no estrus = 16.2% [89/562], estrus = 26.5% [165/628]) and 60 days (no estrus = 13.3% [71/562], estrus = 23.1% [143/628]) pregnancy diagnosis, independent of

treatment. There was a tendency for greater pregnancy loss from 32 to 60 days in cows that did not show estrus (no estrus = 19.7% [18/89] as compared to those that showed estrus = 12.4% [22/165]) independent of treatment. In cows that had their estrous cycle synchronized, expression of estrus was associated with an increase in P/AI at 32 days (no estrus = 20.9% [46/220], estrus = 28.4% [90/317]) and 60 days (no



estrus = 17.3% [38/220], estrus = 24.3% [77/317]) pregnancy diagnosis, independent of treatment. No difference was observed for pregnancy loss from 32 to 60 days for cows detected or not in estrus (estrus = 13.2% [13/90]; no estrus = 16.5% [8/46]).

Experiment 3 (Pereira *et al.*, Department of Animal Production, FMVZ-UNESP, Botucatu, SP, Brazil; unpublished data) was designed to determine whether increasing the length time interval with reduced circulating P4 (proestrus) would increase fertility in a TAI program that utilized E2 and P4 to synchronize ovulation of cycling, lactating dairy cows. The study used a total of 759 lactating Holstein cows. Within each farm (n = 3), cows were blocked by parity (primiparous and multiparous), all non-pregnant cows that had passed

the voluntary waiting period for the farm were utilized and randomized into the study, without regard to whether they had been previously utilized in the study. Within each block, 1,101 cows were scanned to determine the presence of a detectable CL (day -11). Cows with a CL (n = 759) were randomly assigned to receive one of two treatments: 1) an intravaginal progesterone insert containing 1.9 g of P4 (CIDR), and 2.0 mg (i.m.) estradiol benzoate (Estrogin) on day -10, 25 mg (i.m.) dinoprost tromethamine (Lutalyse) on day -3, CIDR withdrawal and 1.0 mg (i.m.) of estradiol cypionate (ECP) on day -2, and TAI on day 0 (treatment 8 days), or 2) CIDR insert + 2.0 mg (i.m.) of EB on day -11, Lutalyse on day -4, CIDR withdrawal + 1.0 mg of ECP on day -2, and TAI on day 0 (treatment 9 days).

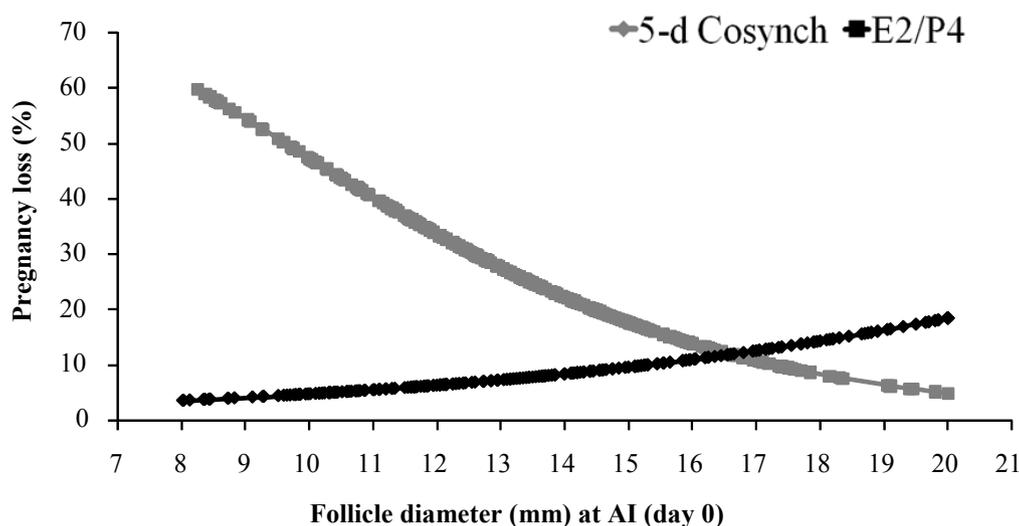


Figure 3. Effect of follicle diameter at AI on pregnancy loss between 32 day and 60 day in cows that had their estrous cycle synchronized ( $P4 \leq 0.4$  ng/ml at day 0 and  $P4 \geq 1.0$  ng/ml at day 7), receiving E2/P4 or 5-day Cosynch protocols.

The use of only lactating cows with a CL at the beginning of the protocol during the cooler months of the year, resulted in a high percentage of cows that responded to the protocols with ovulation (>90%) and high fertility (>40%). We postulated that increasing the length of time from PGF2a treatment to TAI by 1 day would allow more time for follicle development and thereby increase expression of estrus and follicle diameter at the time of AI. Table 4 shows the effect of treatments on detection of estrus, ovulation to the protocol, P/AI at days 32 and 60, and pregnancy loss for lactating dairy cows receiving protocols of 8 or 9 days duration. The cows in the 9 days protocol were more likely to be detected in estrus compared with the 8 days protocol. However, >90% of cows had their estrous cycles synchronized to either protocol with no difference between groups. In cows with their estrous cycle synchronized, both protocols resulted in a high P/AI at days 32 (~48%) and 60 (>40%) of pregnancy, with no difference between protocols. Nevertheless,

increasing the length of the protocol from 8 to 9 days reduced the pregnancy losses that occurred between days 32 and 60 of pregnancy.

Although treatment did not have an effect on average follicle diameter at TAI, expression of estrus (Table 5) increased the percentage of cows with a CL on day 7, increased circulating P4 concentrations on day 7, and increased P/AI at days 32 and 60 of pregnancy in synchronized cows regardless of treatment. There was no interaction detected between expression of estrus and treatment for any of these variables. A greater pregnancy loss from 32 to 60 days was observed in cows that did not show estrus.

Follicle diameter at TAI affected P/AI at the 32 and 60 days pregnancy diagnoses in cows that ovulated to the protocol, independent of whether cows were detected in estrus (Fig. 4). In cows not detected in estrus, the follicle diameter had an effect on pregnancy loss (Fig. 5), however, there was no effect of follicle diameter on pregnancy loss from 32 to 60 days in cows that were detected in estrus.



Table 4. Treatment effects on estrus detection, estrous cycle synchronization, and P/AI at days 32 and 60 after TAI and pregnancy loss in cows receiving an 8 or 9 day synchronization protocol.

Item <sup>1</sup>	Protocol length		P - value
	8 day	9 day	
Estrus detection	63.4 (240/385)	73.0 (269/374)	<0.01
Estrous cycle synchronization <sup>2</sup>	92.8 (352/379)	91.5 (339/370)	0.52
P/AI			
At day 32	45.0 (175/385)	43.9 (166/374)	0.77
At day 60	38.1 (150/385)	40.4 (154/374)	0.52
Pregnancy Loss	14.7 (25/175)	7.6 (12/166)	0.04
P/AI <sup>2</sup>			
At day 32	48.1 (170/352)	47.9 (163/339)	0.96
At day 60	40.5 (145/352)	43.9 (151/339)	0.37
Pregnancy loss	15.2 (25/170)	7.8 (12/163)	0.03

<sup>1</sup>Least squares means % (no./no.). <sup>2</sup>Cows that had their estrous cycle synchronized in response to the protocol (CL at day 7).

Table 5. Treatments effects by estrus detection on distribution, estrous cycle synchronization, P/AI at 32 and 60 day and pregnancy loss from 32 to 60 day in cows that had their estrous cycle synchronized, receiving 8 or 9 day protocol length. Results are reported as least-squares means.

Item <sup>1</sup>	Estrus		P - value
	No	Yes	
Distribution			
8 days	38 (145/385)	62 (240/385)	—
9 days	28 (105/374)	72 (269/374)	—
P - value	<0.01	<0.01	
Ovulatory follicle diameter (mm)	14.6 ± 0.37	14.8 ± 0.33	0.69
Estrous cycle synchronization <sup>1</sup>	81.0 (202/248)	97.4 (489/501)	<0.01
P4 day 7 <sup>2</sup>	2.77 ± 0.17	3.22 ± 0.16	<0.01
P/AI <sup>2</sup>			
At day 32	39.4 (81/202)	51.2 (252/489)	<0.01
At day 60	31.1 (66/202)	46.3 (230/489)	<0.01
Pregnancy loss	19.8 (15/81)	9.3 (22/252)	<0.01

<sup>1</sup>Least squares means % (no./no.). <sup>2</sup>Cows that had their estrous cycle synchronized in response to the protocol (CL at day 7).

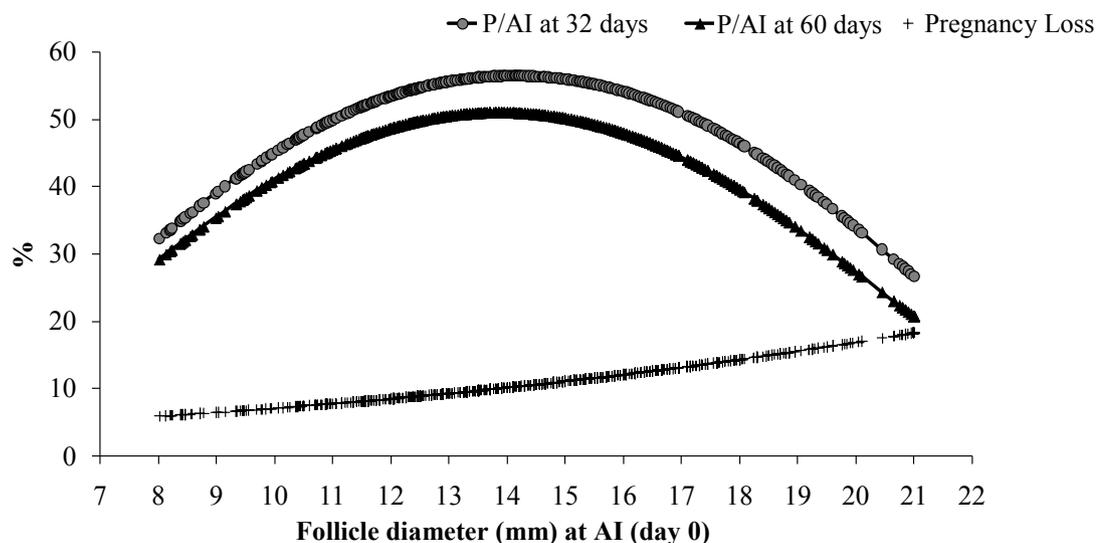


Figure 4. Effect of follicle diameter at day 0 on P/AI at 32 and 60 day and pregnancy loss in dairy cows that had their estrous cycle synchronized in response to the protocol (CL at day 7), receiving 8 or 9 day protocol length. 30 day, P < 0.01; 60 day, P < 0.01; Pregnancy loss, P = 0.15.

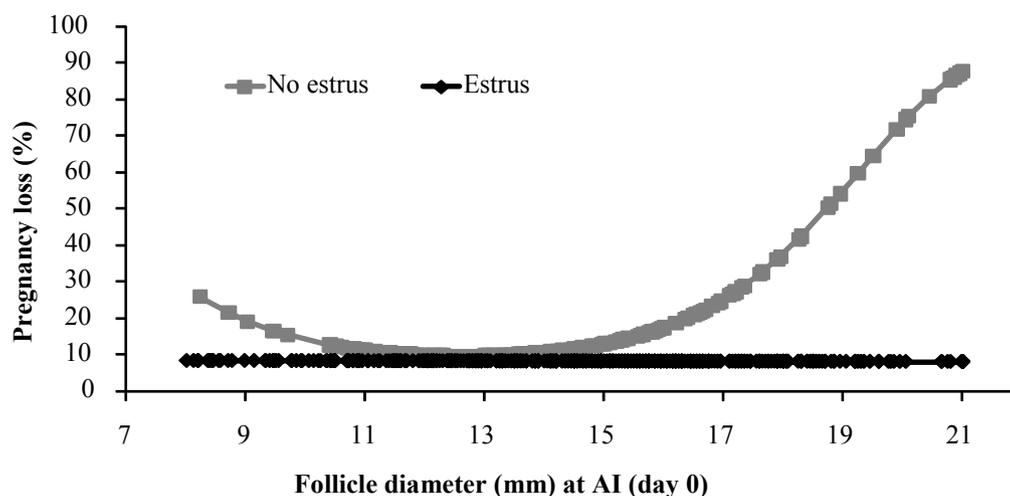


Figure 5. Effect of follicle diameter at AI (day 0) on pregnancy loss between days 32 and 60 in dairy cows that had their estrous cycle synchronized (CL at day 7), receiving an 8 or 9 day synchronization protocol length. No estrus,  $P < 0.01$ ; estrus,  $P = 0.97$ .

### Discussion

In experiment 1, anticipating the timing of the PGF2 $\alpha$  treatment in cycling animals bred by TAI, increased pregnancy. Treatment with PGF2 $\alpha$  on day 7 also reduced P4 concentrations at the time of P4 device removal/ECP treatment 24 h later. It seems that earlier PGF2 $\alpha$  treatment improves fertility to TAI, potentially by increasing period of proestrus and reducing P4 near AI. Some studies have found that increasing the time of proestrus increased fertility. In *Bos taurus* beef cows the premature induction of an LH surge, when follicle diameter reached 10 mm, reduced fertility compared to cows allowed to proceed to spontaneous estrus and ovulation (Mussard *et al.*, 2007). Reducing the proestrus period from 2.25 day to 1.25 day resulted in more short luteal phases (35% [40] vs. 82% [38], respectively), and reduced fertility (50.0 vs. 2.6%, respectively) in another study (Bridges *et al.*, 2010). Earlier treatment with PGF2 $\alpha$  in an E2/P4-based TAI program for beef cattle resulted in significant improvements in fertility (Meneghetti *et al.*, 2009; Peres *et al.*, 2009). Cows with reduced circulating P4 concentrations at the time of P4-intravaginal device removal had higher fertility to TAI than cows with higher P4 at the time of P4-intravaginal device removal (Dias *et al.*, 2009; Meneghetti *et al.*, 2009; Peres *et al.*, 2009). In dairy cattle, reducing the proestrus length from 36 to 0 h during an Ovsynch program reduced P/AI (linearly from 8.8% at 0 h; 13.2% at 12 h; 21.4% at 24 h; 28.0% at 36 h) as the proestrus period increased and with increasing size of the ovulatory follicle, and a tendency for reduced short luteal phases (Peters and Pursley, 2003). In grazing lactating dairy cows presynchronized with the Presynch program ( $n = 1,754$ ), an increase in proestrus period from Cosynch at 56 h to Cosynch at 72 h increased fertility at the 65 day pregnancy diagnosis (54.9 vs.

46.5%) but did not alter fertility at the 30 day pregnancy diagnosis and did not alter fertility at either pregnancy diagnosis in cows presynchronized with Double Ovsynch (Ribeiro *et al.*, 2012). Most of these data are consistent with our results that increasing the length of proestrus increases fertility in TAI programs. It seems likely that increasing the length of proestrus increased E2 concentrations near the time of AI, reduced short luteal phases and increase P4 concentrations after AI (Vasconcelos *et al.*, 2001).

In experiment 2, P/AI were increased with the E2/P4 protocol compared with the 5-day Cosynch protocol. Follicle diameter has been associated with P/AI in a number of studies. Ovulation of small follicles is associated with reduced P/AI, reduced E2 concentration, an increase in the incidence of short luteal phases (Vasconcelos *et al.*, 2001), and increased pregnancy loss (Perry *et al.*, 2005). A primary reason for the reduced P/AI in the 5-day Cosynch protocol was the high pregnancy loss in this group; pregnancy losses between days 32 to 60 of pregnancy was nearly twice as high as in the E2/P4 protocol. The potential explanation for these results is that circulating E2 concentrations were greater in the cows that had ovulation induced with ECP rather than GnRH. Although we did not measure circulating E2 in this trial, the physiological effects of elevated E2 are clearly manifest with greater expression of estrus in the cows in the E2/P4 protocol compared with the 5-day Cosynch protocol. Thus, we speculate that the improved P/AI and reduced pregnancy loss in the cows in the E2-based protocol resulted from increased circulating E2 concentrations near the time of TAI that may have a positive effect on fertilization, embryonic development, and subsequent pregnancy maintenance.

All of these data are consistent with the concept that greater circulating E2 near AI may reduce



pregnancy loss at the later embryonic stages (32 to 60 days after AI). Consistent with this hypothesis, there was an increase in the percentage of cows detected in estrus with the longer compared to the shorter protocol in experiment 3 and detection of estrus was associated with greater synchronization and reduced pregnancy losses between 32 and 60 days. Our main hypothesis was that the longer protocol would result in greater fertility. Although, there was no overall change in P/AI, we observed a decrease in pregnancy loss between 32 and 60 days with the longer protocol. This result highlights the importance of pregnancy loss as a critical reproductive measure in lactating dairy cows, and the association that we detected between pregnancy loss and lack of estrus. Our study does not allow us to determine the mechanism that results in lower fertility and greater pregnancy loss in cows that did not demonstrate estrus. One possibility is that reduced E2 near TAI increases pregnancy loss as demonstrated in a recent study (Roberts *et al.*, 2012). Ovariectomized cows that did not receive E2 in the preovulatory period maintained pregnancy until day 21, however, by day 29 reduced pregnancies were detected when compared to cows that received either ECP or EB to simulate the preovulatory period. In our study, ECP was given to increase circulating E2 in the preovulatory period; however, additional E2 from the follicle may be required to produce estrus and an optimal uterine environment. Alternatively, a greater time for CL regression and reduced circulating P4 near TAI, may increase expression of estrus and potentially improve pregnancy maintenance by enhancing the uterine environment. Development of more optimized protocols, potentially demonstrated by increased expression of estrus, should improve P/AI and reduce pregnancy losses.

In a recent study, single embryos were transferred into recipient beef cows induced to ovulate either small or large follicles using GnRH (Atkins *et al.*, 2013; Jinks *et al.*, 2013). Concentration of E2 at GnRH treatment in the recipient cows was one of the most important factors that determined pregnancy outcome (Atkins *et al.*, 2013). Similarly, circulating E2 at GnRH-induced ovulation in the recipient cows, but not the donor cows, was predictive of pregnancy success at day 27 of gestation (Jinks *et al.*, 2013). Further, administration of ECP 24 h before expected time of AI in recipients, increased pregnancy success in cows induced to ovulate a small dominant follicle (<12.2 mm). Thus, the primary benefit of increased preovulatory E2 is mediated through alterations in the maternal environment of the recipient cows. Whether inadequate E2 is responsible for reduced success in lactating dairy cows ovulating small follicles following an ECP-induced ovulation remains to be determined.

Alternatively, ovulation of very large follicle can also be associated with reduced fertility, possibly because of excessive length of dominant follicle

persistence (Townson *et al.*, 2002; Bleach *et al.*, 2004; Cerri *et al.*, 2009). An interesting paradox is that although increased circulating P4 after AI can improve fertility (Demetrio *et al.*, 2007; Forro *et al.*, 2012; Wiltbank *et al.*, 2012), cows that ovulate larger follicles (>17 mm) had greater P4 concentrations on day 7 but lower P/AI at 60 day, compared to cows that ovulate follicles between 11 to 17 mm.

## Conclusions

Circulating concentrations of progesterone and estradiol prior to and circulating concentrations of progesterone following ovulation can affect fertility in cattle. In addition, small increases in P4 concentrations near the time of AI, due to lack of complete CL regression, result in reductions in fertility. Earlier treatment with PGF2 $\alpha$  should allow greater time for CL regression, an increase in estradiol, and subsequent reductions in circulating P4 that could be critical for fertility. Optimization of follicle size in TAI programs is clearly an intricate balance between oocyte quality, adequate circulating E2 near AI, and adequate circulating P4 after AI.

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## Doppler ultrasonography as a tool for ovarian management

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### Abstract

Doppler ultrasonography allows the characterization and measurement of blood flow, and can be used to indirectly make inference regarding the functionality of different organs, including the ovaries. Several studies highlighted the importance of an adequate blood flow for follicle development and acquisition of ovulatory potential, and for progesterone secretion by the corpus luteum. Due to some particularities of the ovarian vascular anatomy, however, different strategies had to be developed to measure the blood flow detected by Doppler imaging. Some of these approaches were successful to characterize the patterns of blood flow throughout the estrous cycle, pregnancy, and early postpartum, but require post-acquisition image processing and do not allow real time decisions to be taken. The subjective evaluation of blood flow has been alternatively used in field conditions aiming to early detect non-pregnant animals, select embryo recipients, or predict artificial insemination and *in vitro* fertilization outcomes. In summary, color-Doppler imaging provides important information about the function of corpus luteum and follicles, supporting clinical diagnoses and management decisions. Nonetheless, the adoption of Doppler ultrasonography as a routine exam, instead of a limited use in an individual basis, requires further development of feasible blood flow evaluation procedures and the establishment of reference values.

**Keywords:** blood flow, cattle, reproduction, vascular dynamics.

### Introduction

The development of image technologies significantly contributed to advances in the field of biological sciences. The possibility of a non-invasive, real time approach to assess tissues and organs was of great importance for the study of many physiological mechanisms and for the diagnosis of pathological conditions. For these purposes, several of technologies, from the X-ray to the modern computer tomography, have been used in human and veterinary medicine. The

B-mode ultrasonography, for example, had a central role in the characterization of ovarian follicle dynamics in different domestic species and in the subsequent development of several protocols to control ovarian function for assisted reproductive technologies (ARTs) such as timed artificial insemination, superovulation, and oocyte pick-up (Adams *et al.*, 2008). The versatility and the number of potential use have made the B-mode ultrasound a valuable and widespread adopted tool in animal reproduction sciences.

Over the past few decades, various new technologies for image diagnosis with potential use in reproductive medicine emerged, including the Doppler ultrasonography (King, 2006). The Doppler technology allows the identification of movement of cells or tissues and is particularly important in the characterization and measurement of blood flow. The vascular dynamics is very closely related to the function of different organs, including those from the reproductive tract, and consequently is an important evaluation parameter of reproductive function. Doppler ultrasonography is routinely used in human gynecology and obstetrics, frequently as the diagnostic gold standard, and also to predict *in vitro* fertilization outcomes (Chui *et al.*, 1997; Borini *et al.*, 2001). The use of Doppler ultrasonography in animal reproduction research is more recent but not less important, and several studies have demonstrated the relationship of blood flow and ovarian and uterine function throughout the estrous cycle and pregnancy (Acosta *et al.*, 2002, 2003; Miyamoto *et al.*, 2005; Honnens *et al.*, 2008; Herzog *et al.*, 2010). In spite of its potential usefulness for reproductive management (Miyamoto *et al.*, 2006; Matsui and Miyamoto, 2009), and differently from the human medicine, the adoption of Doppler imaging technology in large animal practice is still limited.

Two main factors have contributed to this low use of Doppler technology in cattle: 1) the availability and cost of portable devices, and 2) the relative lack of knowledge by practitioners about its potentials as a tool to evaluate reproductive function. Along with the recent technological advances in electronics and data processing computers there is a trend for the reduction in both size and price of portable ultrasound devices (Herzog and Bollwein, 2007). The present article aims

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to review some of the particularities of the use of Doppler ultrasonography in the evaluation of ovarian function and its implications for cattle reproductive management, considering the basic mechanisms of the technique, the vascular physiology of the ovary, and the alternatives to evaluate its vascularization.

### **Doppler ultrasonography: basic principles, limitations, and alternatives for ovarian evaluation**

The Doppler Effect was first described in 1842 by Christian Johan Doppler. It is a natural phenomenon characterized by the apparent change in the sound wave frequency when the source of the wave moves towards or away from the receptor. The difference between the frequency generated and received (Doppler shift) is proportional to the velocity of the movement. This effect also occurs with ultrasound waves, and the frequency difference caused by the movement of blood cells allows for the detection and measurement of blood flow (Ginther, 2007). The change in the frequency of reflected waves relative to emitted waves can be positive, when blood cells move towards the transducer; or negative, when cells move away from the transducer (Herzog and Bollwein, 2007).

The Doppler shift can be represented in the ultrasound screen as a colored image (color and power Doppler) or as a graph (spectral Doppler). In the color flow mode, the Doppler shift is shown as a spectrum of one or two colors, usually red and blue, which represents the direction of blood flow. In the power flow mode the color represents the intensity of the Doppler signal, regardless of direction. In the spectral flow mode, the Doppler shift is represented as a chart (speed by time). In an artery, for example, the spectral graph will typically have a wave form corresponding to the arterial pulse in each cardiac cycle (systole and diastole), while in veins the flow will be almost constant, without a pulse pattern (Herzog and Bollwein, 2007). The spectral flow mode allows a detailed analysis of flow distribution and waveform, and consequently the calculation of velocity and indices (pulsatility index [PI], resistance index [RI]), in one particular vessel. The color flow mode, on the other hand, provides less flow information, but allows an overview of the blood flow perfusion in a region.

Most of the objective measurements of blood flow performed by Doppler ultrasonography, including calculation of flow velocity and indices, are usually performed in large and straight arteries and veins. However, the evaluation of blood flow in the genital system, especially the ovary may be particularly challenging. Arterioles and venules detectable by ultrasound in follicles and corpora lutea represents only part of the vascular plexuses of these structures (Jiang *et al.*, 2003), and besides having very small diameters they show slow blood flow, requiring the Doppler function to be set for a high sensitivity (usually by decreasing pulse repetition frequency [PRF]). This high sensitivity,

however, requires a well-immobilized patient during the exam, what is frequently difficult to achieve in large animals. The interference of breathing and peristaltic movements may also impair correct caliper positioning during the exam and so spectral mode Doppler is hardly used in routine evaluations of cattle ovaries. Additionally, the Doppler shift is affected by the angle (insonation angle) between the Doppler beam and the flow direction; therefore calculation of blood flow velocity requires the knowledge of the vessel orientation (Ginther, 2007). Besides being small, most of the vessels in the follicular wall or around the corpus luteum are tortuous, and it is difficult to establish the insonation angle and, consequently, to have a correct estimation of flow velocity. Finally, the architecture of the ovarian artery, which is coiled around the ovarian vein, reduces the pulse pattern of the ovarian arterioles, affecting PI and RI.

To overcome these limitations, some alternative approaches have been used for the assessment of blood flow in follicles and corpora lutea. One of the most used is the measurement of the colored area in one or a few representative images of the area under study (Acosta *et al.*, 2002; Ginther *et al.*, 2007; Rauch *et al.*, 2008). In this approach, the ovarian structure is scanned and the color-Doppler mode is used to identify the blood flow, and cross section images of the central area or of the areas with greatest Doppler signal are recorded and later measured using software for image analysis. Alternatively, power mode Doppler can be used and pixel analysis performed in the colored areas to have a semiquantitative assessment of luteal or follicle blood flow (Lüttgenau *et al.*, 2011a, b). This methodology is relatively simple to perform and results are usually coherent with the expected physiological variation in blood flow (Herzog *et al.*, 2010). Unfortunately, images post-processing prevents real-time decisions to be taken. Although a high repeatability was reported for blood flow evaluation based on single images (Lüttgenau *et al.*, 2011b), bias may also occur due to the unevenly distribution of angiogenesis, especially in the follicle, where largest vessels are present mainly in the basal region (Jiang *et al.*, 2003). During transrectal ultrasound exam of the ovaries, it is not always possible to adjust the angle of scanning in order to have in the same (“representative”) picture the cross section of a follicle on its maximum diameter and on the area with the maximum number of colored pixels. This could explain, to some extent, the relatively low correlation between follicle blood flow (FBF) and follicle diameter observed during dominant follicle growth (Lüttgenau *et al.*, 2011a; Arashiro *et al.*, 2012), comparing to that observed between corpus luteum blood flow (CLBF) and corpus luteum (CL) size.

Another approach routinely used by both researchers and practitioners is the subjective evaluation of Doppler images (Ghetti *et al.*, 2012). In this methodology the technician ranks blood flow based on a



general overview of the Doppler signal in all areas of the structure, based on a mental reconstruction of the three-dimensional image of the vascularization. When there is few Doppler signal, as occurs in small follicles, blood flow can also be simply classified as “present” or “absent” (Pancarci *et al.*, 2012). The subjective approach is a fast and straightforward way to evaluate blood flow, in which diagnostic and decisions can be taken during the exam and, consequently, is suitable to most of the field reproductive management routines. The subjective approach, as any other methodology based on visual ranking instead of objective measurement, is more difficult to standardize and requires previous experience of the evaluator (Siqueira *et al.*, 2013). Moreover, it will be more efficient when the amount of detectable blood flow is larger, as occurs when we evaluate blood flow in the CL, in comparison to evaluation in follicles; or when the expected differences are greater, as occurs in day-20 CL between pregnant and nonpregnant cows. Previous studies, however, reported a good correlation of blood flow between objective and subjective methods, for both follicles and CL ( $R = 0.72$  and  $R = 0.78$ , respectively;  $P < 0.0001$ ), in a single evaluation (Areas *et al.*, 2012; Ghetti *et al.*, 2012). Figure 1 illustrates the subjective and objective approaches to evaluate the CL.

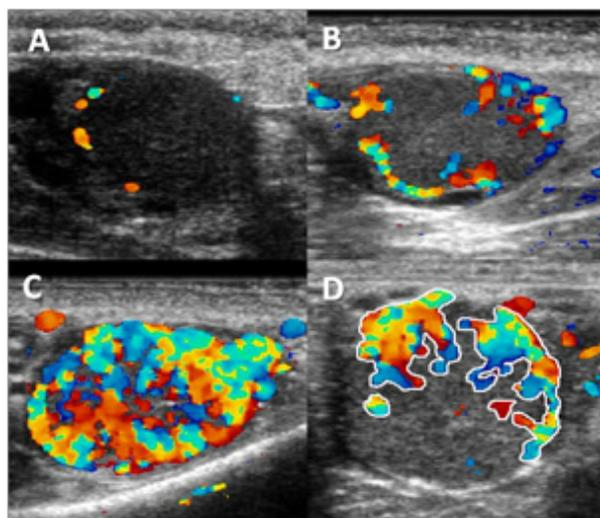


Figure 1. Corpus luteum blood flow evaluation with color flow Doppler imaging. A-C: Corpus luteum subjectively scored as presenting low (A), intermediate (B) or high (C) vascularization. D: Corpus luteum with Doppler signal (colored) area delimited (in white) for further objective measurement with image assisted computer analysis.

Three-dimensional (3D) modeling was alternatively used to objective evaluation of blood flow volume in corpora lutea (Jokubkiene *et al.*, 2006) and follicles (Lozano *et al.*, 2007) in human and, more recently, in cattle (Arashiro *et al.*, 2012). This new approach allowed an objective measurement of blood

flow, using a set of images that covers the whole structure under analysis. Besides, it is possible to reconstruct the vascular architecture of follicles and CL, what was previously done only with invasive methodologies such as vascular polymer injection and subsequent corrosion of organic tissues (Jiang *et al.*, 2003). The 3D modeling approach also requires post-acquisition image processing and therefore is time-consuming, but may open new possibilities for ovarian vascular dynamics studies. Figure 2 shows 3D models of a CL on day 12 of the estrous cycle with a typical distribution of blood flow (from the periphery to the center; [2B]), and a CL with an unusual vascular architecture (from the center to the periphery; [2D]). Although this uncommon blood flow pattern, the cow bearing this CL was later diagnosed as pregnant (personal data).

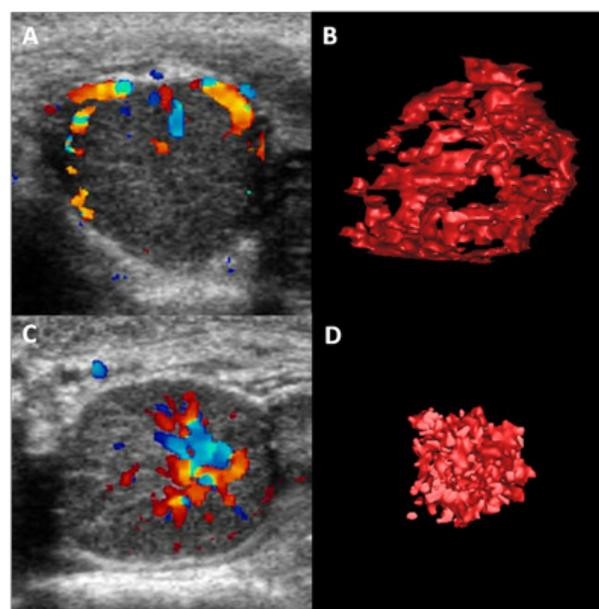


Figure 2. Three-dimensional modeling of corpus luteum vascularization. A-D: Color flow Doppler imaging (A, C) and respective 3D models (B, D) of a corpus luteum with a typical distribution of blood flow (from the periphery to the center; A, B), and of a corpus luteum with an unusual vascular architecture (from the center to the periphery; C, D).

Computer-assisted ultrasound image analysis was first reported for the evaluation of CL and follicle echotexture (Singh *et al.*, 2003), and is a requirement for most studies on the objective blood flow evaluation in both 2D and 3D Doppler imaging. Different computer programs were used in ovarian Doppler evaluation, including open-source (Image-J, Acosta *et al.*, 2002) and commercial versions of general purpose (Adobe Photoshop, Acosta *et al.*, 2002; FixFoto, Lüttgenau *et al.*, 2011b; Mimics, Arashiro *et al.*, 2012) or custom image analysis software (PixelFlux, Rauch *et al.*, 2008; VOCAL, Jokubkiene *et al.*, 2006). Software



choice must consider the goals and complexity of each study, the Doppler ultrasound device available, as well as the type and resolution of the image files generated.

### Vascularization and ovarian function

The development of the ovarian follicle is closely related to the development of a vascular network in the theca layer, which provides nutrients, hormones and oxygen to support follicle growth and steroidogenesis. There are no blood vessels in the granulosa layer and metabolites reach the intrafollicular environment by diffusion. The vascular plexus of medium and large follicles are supplied by individual spiral arteries but drained by several veins (Jiang *et al.*, 2003). There are many evidences demonstrating the relationship between vascularization and follicular function. Angiogenesis and ischaemia are related to the fate of follicles, although it is not clear whether they are cause or consequence of follicle development or atresia (Jiang *et al.*, 2003). Blood flow affects the composition of follicular fluid (Rodgers and Irving-Rodgers, 2010) and is positively correlated to intrafollicular estradiol concentrations and estradiol:progesterone ratios (Pancarci *et al.*, 2012). Follicular blood flow increases during follicle deviation (Arashiro *et al.*, 2012) and reaches a peak in the preovulatory follicle after the onset of the LH surge or 0.5 h after GnRH injection (Acosta *et al.*, 2003), supporting the concept that a close association between blood flow and the morphological and functional changes precedes ovulation. Consequently, FBF affects oocyte quality and its developmental potential (Sutton *et al.*, 2003). Follicle vascularization is also associated to responsiveness to exogenous stimulation and to further embryo implantation rates (Lozano *et al.*, 2007), and has been used in human medicine to predict *in vitro* fertilization outcomes (Chui *et al.*, 1997).

The CL is highly vascularized and shows the greatest blood flow per unit of tissue in the body (Acosta *et al.*, 2002). Luteogenesis is characterized by intensive angiogenesis, and CLBF increases 3 to 4-fold in the first 96 h after ovulation (Acosta *et al.*, 2003). Most of steroidogenic cells are adjacent to one or more capillaries (Reynolds *et al.*, 2000) and luteal blood flow perfusion is so intense that affects acoustic impedance of the organ, resulting in the hypoechoic echotexture characteristic of the CL (Singh *et al.*, 1997). Neovascularization during CL development is important to support the rapid growth of luteal tissue and is essential for both the provision of substrate for progesterone biosynthesis and for the delivery of steroids to the general circulation (Miyazaki *et al.*, 1998). Plasma progesterone concentrations are positively correlated to CLBF throughout the estrous cycle, reflecting the development and regression of luteal tissue (Herzog and Bollwein, 2007). Progesterone secretion is the gold standard for functional evaluation

of the CL, particularly during luteolysis, when changes in luteal tissue volume are slower and more progressive than in luteal function (Niswender *et al.*, 1994). Coherently, the correlations between CLBF or progesterone and CL size are not as strong as between CLBF and plasma progesterone, demonstrating that Doppler ultrasonography improves the accuracy of diagnoses by adding physiological information from the organ (Herzog *et al.*, 2010). Interestingly, both natural and PGF2 $\alpha$ -induced luteolysis are characterized by an initial acute increase in blood flow, followed by a significant drop 24 to 48 h later (Ginther *et al.*, 2007). This initial rise is associated with a transient vasodilation in peripheral vessels of the CL, driven by an increase in endothelial nitric oxide synthase stimulated by the PGF2 $\alpha$  (Shirasuna *et al.*, 2008), and must be taken into account as a factor affecting single evaluations of the CLBF performed during the expected period of luteolysis, specially when the goal is to early identify non-pregnant animals (Siqueira *et al.*, 2013).

### Color flow Doppler imaging in the reproductive management

Color flow Doppler imaging can be used to assess the amount and pattern of blood flow within the ovary, and indirectly indicates its functionality. This is particularly useful to characterize the presence of luteal tissue, as in early developing CL, tumors, or in luteinized cysts (Rauch *et al.*, 2008), and to make indirect inference regarding CL function, i.e., progesterone secretion. Visualization of CLBF may therefore be used either in studies of ovarian physiology or for reproductive management decisions. A CL that does not present enough Doppler signals (colored pixels), an evidence of lack of blood supply, may be considered as not functional; the same CL, however, may present a normal size (diameter or area). From this point of view, gray-scale ultrasonography is limited to differentiate from a functional vs. a nonfunctional CL, if they have similar size. This type of assessment of functionality is only possible by using Doppler imaging. Indeed, blood flow has been suggested to be more appropriate than size for CL function evaluation (Herzog *et al.*, 2010), because CL vascularization plays a key role in regulating luteal function (Miyamoto *et al.*, 2005). Evaluations of CLBF could, consequently, be useful to detect pregnancy failures or to predict pregnancy rates after embryo transfer or timed-AI.

In cattle and other domestic mammals, a functional CL is required for pregnancy establishment, because progesterone supports preimplantation embryonic development, allowing maternal recognition of pregnancy (Mann and Lamming, 1999). Failure in conception or the presence of a non-viable embryo results in absence of maternal recognition of pregnancy, which will consequently lead to CL regression (reviewed by Niswender *et al.*, 2000). Luteolysis is



primarily associated to a progressive decrease in the blood flow to the CL in response to prostaglandin F2 $\alpha$  and luteal oxytocin. Although the CL's loss of function (progesterone secretion) responds rapidly to the decrease in blood flow, its morphological regression (reduction in size) only becomes evident hours later (Niswender *et al.*, 1994). This asynchrony between functional and morphological changes limits the usefulness of CL evaluation by conventional B-mode ultrasonography, based on the evaluation of CL size (diameter or area), to determine luteal function around the time of luteolysis (Kastelic *et al.*, 1990). The use of color-Doppler ultrasound can overcome these limitations because it allows real-time CLBF assessment, an indirect measure of CL functionality, especially by the end of the estrous cycle (Miyamoto *et al.*, 2006; Herzog *et al.*, 2010). In the reproductive management routine, an important strategy to improve reproductive efficiency is to detect failure in conception as early as possible. It has been suggested that color-Doppler imaging could be used to early detect nonpregnant animals, by evaluating CLBF (Matsui and Miyamoto, 2009; Siqueira *et al.*, 2013).

Although gray-scale ultrasonography has been successfully used for transrectal early pregnancy diagnosis in cattle (Pierson and Ginther, 1984), this approach presents low reliability and accuracy when performed earlier than 26 days post AI (Pieterse *et al.*, 1990) because clear visualization of the embryo/vesicle in the uterine lumen at this stage is difficult and time consuming (Quintela *et al.*, 2012). Therefore, considering that a functional CL is mandatory for pregnancy establishment, the lack of CLBF on specific days of the estrous cycle may be used as a direct indicator of nonpregnancy. In fact, previous studies have already suggested that a decrease in CLBF around 19 to 21 days post AI is an indicator of nonpregnancy (Matsui and Miyamoto, 2009) and also that color Doppler flow imaging could be useful for an accurate early pregnancy diagnosis in cattle (Matsui and Miyamoto, 2009; Quintela *et al.*, 2012). Briefly, the idea is to detect, by color-Doppler imaging, normalized, non-functional corpora lutea, which indicates failure in conception. Results of these types of approaches are still controversial, and some authors recommend CLBF for early pregnancy diagnosis (Matsui and Miyamoto, 2009; Quintela *et al.*, 2012) but others reported low accuracy (Utt *et al.*, 2009) and reliability (Herzog *et al.*, 2011) of this diagnostic method. Perhaps, the explanation for this apparent disagreement lays on the fact that CLBF evaluation has been performed at different days of the estrous cycle in those studies. A recent report showed high accuracy and reliability of pregnancy diagnoses performed on day 20 post AI, using subjective evaluation of CLBF as a unique criterion (Siqueira *et al.*, 2013). The subjective evaluation of CLBF is a feasible approach, and its adoption in the reproductive routine may allow for the

early resynchronization of nonpregnant animals and consequent reduction in days open and calving intervals.

In embryo transfer programs, synchronized recipients are routinely selected based on the presence and quality of the CL on days 7 or 8 of the estrous cycle. The relationship between CL quality, assessed by size or echotexture, and pregnancy rates have been evaluated after the transfer of embryos produced *in vivo* or *in vitro* (Siqueira *et al.*, 2009). Although there is a general consensus that CL function affects the likelihood of pregnancy, the relatively poor association between CL morphological characteristics and progesterone production limits the predictive value of B-mode ultrasonography for recipient selection. Progesterone secretion and CL blood flow are positively correlated throughout the estrous cycle (Herzog *et al.*, 2010) and therefore color-Doppler ultrasonography could be a valuable tool to select embryo recipients. Our preliminary results (Guimarães *et al.*; Universidade José do Rosário Vellano, Alfenas, MG; unpublished data) however, showed no differences in CLBF between embryo recipients later diagnosed as pregnant or nonpregnant. This apparent contradiction may be caused by the complex interplay among embryo quality, embryo developmental stage, and endometrial timing by progesterone (Loneragan, 2011). Although the role of progesterone in the establishment and maintenance of pregnancy is well known (Mann and Lamming, 1999), the minimum threshold for the establishment of pregnancy is controversial (Spell *et al.*, 2001) and consequently it is difficult to determine a cutoff value. Moreover, in spite of characterizing the association of CLBF and progesterone during the estrous cycle (Herzog *et al.*, 2010), the same group observed that single Doppler evaluations during mid-luteal phase (on day 9 of the estrous cycle) failed to correlate CLBF and plasma P4 concentration (Lüttgenau *et al.*, 2011b). Thus, it is likely that Doppler evaluation, as occurs with CL size and even with progesterone concentration, will provide additional information to interpret further pregnancy rates after embryo transfer, but may be of limited use as a tool to select recipients.

Follicular blood flow evaluation has been used to predict follicle status and oocyte quality in human medicine (Sutton *et al.*, 2003; Lozano *et al.*, 2007). In cattle, there are some studies highlighting the importance of vascularization on follicle viability, growth, ability to become dominant and ovulate (Acosta *et al.*, 2003; 2005; Pancarci *et al.*, 2012), but few reports associating vascularity of preovulatory follicles and subsequent fertility (Siddiqui *et al.*, 2009a). Although evidences of a direct relationship between blood flow and the fate of the follicle were previously reported (Acosta *et al.*, 2005), the use of FBF in a reproductive routine still requires the establishment of feasible evaluation procedures and respective reference values or criteria. For example, FBF can be detected from small (4-6 mm) to preovulatory follicles and this information



can be used to predict oocyte quality and *in vitro* embryo production (Siddiqui *et al.*, 2009b; Pancarci *et al.*, 2012). In the commercial embryo production laboratories, however, oocytes are not tracked individually throughout the process and therefore embryos cannot be linked to the status of the follicle of origin. A single exam of FBF can be performed in preovulatory follicles, for example as selection criteria to decide for insemination. Follicles presenting no Doppler signal are probably undergoing atresia (Acosta *et al.*, 2005) and will be related to conception failures. In most cases, however, preovulatory follicles will present some degree of vascularization (Ghetti *et al.*, 2012) and the possibility of using these differences to predict fertility is controversial. Actually, there is a significant variation in other predictors of follicle status, such as in intrafollicular concentrations of estradiol, even among growing follicles of the same diameter and presenting estradiol:progesterone ratios higher than 1 (Viana *et al.*, 2013). The physiological relevance of this variation is unclear, and consequently the same is possible for minor differences in FBF.

In summary, color Doppler imaging provides important information about the function of CL and follicles, supporting clinical diagnoses and management decisions. For example, the technology can be used for the characterization of luteal tissue or for the early detection of luteolysis and prediction of pregnancy failures. Nonetheless, the adoption of Doppler ultrasonography as a routine exam, instead of a limited use in an individual basis, still requires further development of feasible blood flow evaluation procedures and the establishment of reference values for a broad range of uses.

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## Role of diestrus progesterone on endometrial function and conceptus development in cattle

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### Abstract

Successful growth and development of the post-hatching blastocyst and pregnancy establishment are a result of the interaction between a competent embryo and a receptive uterine environment. Progesterone (P4) plays a key role in reproductive events associated with establishment and maintenance of pregnancy through its action on the uterine endometrium. Elevated concentrations of circulating P4 in the immediate post-conception period have been associated with an advancement of conceptus elongation, an increase in interferon-tau production and higher pregnancy rates in cattle. The potential beneficial effects of exogenous P4 supplementation on fertility have been acknowledged for a long time but results of supplementation have been inconsistent and may be related to the strategy used to achieve high P4 endogenous concentrations in the animal. This review summarizes recent data highlighting the role of progesterone in regulating uterine function and embryo development in cattle.

**Keywords:** conceptus elongation, cow fertility, embryo mortality, maternal recognition of pregnancy.

### Introduction

The steroid hormone progesterone (P4) plays a key role in reproductive events associated with establishment and maintenance of pregnancy. Conceptus growth and development require the action of P4 on the uterus to regulate endometrial function, including conceptus-maternal interactions, pregnancy recognition, and uterine receptivity to implantation. A considerable proportion of embryo loss may be attributable to inadequate circulating P4 concentrations and the subsequent downstream consequences on endometrial gene expression and histotroph secretion into the uterine lumen. Indeed, low P4 concentrations have been implicated as a causative factor in the low pregnancy rates observed in high-yielding dairy cows (Diskin and Morris, 2008). Elevated concentrations of circulating P4 in the immediate post-conception period have been associated with an advancement of conceptus elongation (Garrett *et al.*, 1988; Satterfield *et al.*, 2006; Carter *et al.*, 2008), an increase in interferon-tau production (Mann and Lamming, 2001), and higher

pregnancy rates in cattle and sheep (Ashworth *et al.*, 1989; Stronge *et al.*, 2005; McNeill *et al.*, 2006). This review summarizes recent data highlighting the role of progesterone in regulating uterine function and embryo development in cattle. The reader is also referred to other recent reviews of the topic (Inskeep, 2004; Spencer *et al.*, 2007; Lonergan, 2011; Wiltbank *et al.*, 2011; Forde and Lonergan, 2012).

### Interaction between the developing embryo and the oviduct

Despite, clear evidence of an interaction between the developing conceptus and the uterine endometrium in early pregnancy (see below), the evidence for reciprocal cross-talk during the transit of the embryo through the oviduct is less clear. Temporal changes occur in the oviduct epithelium gene expression during the estrous cycle (Bauersachs *et al.*, 2004) reflecting the changing requirements of the embryo. There is very convincing evidence for an effect of the oviduct on the quality of the early embryo. For example, short term culture of in vitro produced zygotes in the oviducts of sheep (Enright *et al.*, 2000; Rizos *et al.*, 2002), cattle (Tesfaye *et al.*, 2007) or even mice (Rizos *et al.*, 2007; Rizos *et al.*, 2010b) has been shown to improve embryo quality measured in terms of morphology, gene expression, cryotolerance and pregnancy rate after transfer. In contrast, relatively little evidence exists of an effect going the other way (embryo to oviduct). The limited data reporting an effect of gametes on the oviduct come from litter-bearing species, where any effect is likely to be amplified (Lee *et al.*, 2002; Fazeli *et al.*, 2004; Alminana *et al.*, 2012). We have recently characterized the transcriptome of the bovine oviduct epithelium at the initiation of embryonic genomic activation on day 3 post estrus in pregnant and cyclic heifers (Maillo *et al.*, 2013, School of Agriculture and Food Science, Dublin; unpublished results). The isthmus region, from which all 8-cell embryos and unfertilized oocytes were collected were compared. While large differences in gene expression were observed between the isthmus and ampulla, preliminary data suggest that the presence of an 8-cell embryo had no effect on the transcriptome of the isthmus, although a local effect at the precise position of the embryo cannot be ruled out.

Hugentobler *et al.* (2010) characterized the effects of changes in systemic P4 (achieved by infusion

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of P4) on amino acid, ion and energy substrate composition of oviduct and uterine fluids on days 3 and 6, respectively, of the estrous cycle in cattle. Progesterone increased uterine glucose, decreased oviduct sulphate and, to a lesser degree, oviduct sodium, but had no effect on any of the ions in the uterus. The most marked effect of P4 was on oviducal amino acid concentrations; 9 of 20 amino acids increased following supplementation, with glycine showing the largest increase of approximately two-fold whereas in the uterus only valine was increased.

### Interdependency of the embryo and reproductive tract

Up to the blastocyst stage, the embryo is somewhat autonomous (i.e., does not need contact with the maternal reproductive tract) as evidenced by the fact that blastocysts can be successfully developed *in vitro* in large numbers using IVF technology and transferred to synchronized recipients. Furthermore, based on the same evidence and the fact that embryos recovered from superovulated donors are typically transferred to non-pregnant synchronized recipients, the reproductive tract does not need exposure to the embryo prior to day 7 (and even up to day 16; Betteridge *et al.*, 1980) in order for a pregnancy to be established. In contrast, the post-hatching and pre-implantation conceptus is dependent on substances in the uterine lumen, termed histotroph, that are derived from the endometrium, particularly the uterine glands, for growth and development. This is demonstrated by the fact that: (i) post-hatching elongation does not occur *in vitro* (Brandão *et al.*, 2004; Alexopoulos *et al.*, 2005); and (ii) the absence of uterine glands *in vivo* results in a failure of blastocysts to elongate (Gray *et al.*, 2002; Spencer and Gray, 2006).

On the maternal side, preparation of the uterine luminal epithelium for attachment of trophoblast and implantation in all studied mammals, including ruminants, involves carefully orchestrated spatio-temporal alterations in gene expression within the endometrium. In both cyclic and pregnant animals, similar changes occur in endometrial gene expression up to initiation of conceptus elongation (approximately day 13), suggesting that the default mechanism in the uterus is to prepare for, and expect, pregnancy (Forde *et al.*, 2011b). Indeed, as mentioned above, it is possible to transfer an embryo to a synchronous uterus 7 days after estrus and establish a pregnancy, as is routine in commercial bovine embryo transfer. It is only in association with maternal recognition of pregnancy, which occurs on approximately day 16 in cattle, that significant changes in the transcriptomic profile are detectable between cyclic and pregnant endometria (Forde *et al.*, 2011b; Bauersachs *et al.*, 2012), when the endometrium responds to increasing interferon-tau (IFNT) secreted by the filamentous conceptus.

### Effect of progesterone on the endometrium and consequences for the embryo

In recent years we and others have made significant progress in clarifying the role of the maternal environment, in particular the role of diestrus progesterone, in the successful establishment of pregnancy in cattle. We have demonstrated that:

- Significant changes occur in the endometrial transcriptome during both the estrous cycle and early pregnancy in cattle (Forde *et al.*, 2009, 2011a, b). As mentioned above, these temporal changes occur irrespective of pregnancy status until the time of maternal recognition of pregnancy when conceptus-induced changes in endometrial gene expression are detectable (Forde *et al.*, 2011b; Bauersachs *et al.*, 2012).
- Elevated P4 results in advancement in the normal temporal changes that occur in the endometrial transcriptome (Forde *et al.*, 2009) and in the timing of P4 receptor downregulation in the luminal epithelium (Okumu *et al.*, 2010), the consequence of which is advancement in conceptus elongation (Carter *et al.*, 2008) that is associated with greater embryonic survival.
- Using a combination of *in vitro* embryo production and *in vivo* embryo transfer techniques, we have shown that the effect of P4 on conceptus development is mediated exclusively via the endometrium (Clemente *et al.*, 2009). Addition of P4 to culture medium had no effect on blastocyst formation (Clemente *et al.*, 2009; Larson *et al.*, 2011) or elongation after transfer to synchronized recipients (Clemente *et al.*, 2009). Most convincingly, the embryo does not need to be present in the uterus during the period of P4 elevation in order to benefit from it, strongly suggesting that the effect of P4 is via the endometrium and altered histotroph composition (Clemente *et al.*, 2009).
- Reducing circulating concentrations of P4 results in an alteration in endometrial transcriptome and retarded embryonic development (Forde *et al.*, 2011a, 2012).
- Follicle aspiration just prior to ovulation results in a reduction in CL size and P4 output, decreased expression of *LHCGR* in luteal tissue and a compromised uterine capacity to support conceptus elongation after transfer of *in vitro* produced blastocysts (O'Hara *et al.*, 2012)
- The ability of the oviduct/uterus of the postpartum lactating dairy cow to support early embryonic development is impaired compared to that of the nonlactating heifer (Rizos *et al.*, 2010a) and postpartum nonlactating cow (Maillo *et al.*, 2012) and this is likely due to low concentrations of progesterone in blood and an inadequate luminal environment.



Collectively, these results highlight the importance of an optimal uterine environment to support successful development of the conceptus. However, the role of the developing conceptus itself in eliciting appropriate temporal and spatial changes in the endometrial functions should not be underestimated. For example, two recent key papers provide strong evidence that the endometrium of the cow reacts differently depending on the type of embryo present (Bauersachs *et al.*, 2009; Mansouri-Attia *et al.*, 2009). In other words, embryos of different quality (i.e., with divergent developmental fates) signal differently to the endometrium and in turn elicit a different response in terms of the endometrial transcriptome. In this way, the endometrium can be considered as a biological sensor that is able to fine-tune its physiology in response to the presence of embryos whose development will become altered much later after the implantation process (Mansouri-Attia *et al.*, 2009).

### Strategies for manipulating diestrus progesterone to improve fertility

The potential beneficial effects of exogenous P4 supplementation on fertility have been acknowledged for a long time (see reviews by Inskeep, 2004; Lonergan, 2011; Wiltbank *et al.*, 2011). Several treatments can be used to increase peripheral concentrations of P4 after AI, including those that (i) increase endogenous function of the existing CL (e.g., strategies which promote growth of the dominant follicle before ovulation resulting in a larger CL), (ii) induce accessory CL formation (e.g., hCG or GnRH administration), or those which supplement progesterone directly (e.g., via injection or intravaginal devices). However, data on outcome in terms of pregnancy rate are often conflicting or inconclusive, and may reflect (i) timing of treatment, (ii) that only a proportion of animals with inherently low P4 may benefit from such treatment, or (iii) the lack of sufficient animal numbers and statistical power in many studies.

Dominant follicle size is associated with subsequent CL size (Vasconcelos *et al.*, 2001). Larger CLs secrete more P4 and this has, in some studies, been associated with improved pregnancy rates. Therefore strategies which promote growth of the dominant follicle before ovulation and/or stimulate CL development are likely to increase pregnancy rate (Baruselli *et al.*, 2010). Equine chorionic hormone (eCG) has been incorporated in synchronization protocols in South America for some time and have been reported to improve pregnancy rates following fixed time AI/ET, although results in lactating cows have been less promising than heifers or beef cows (Bo *et al.*, 2011).

Human chorionic gonadotropin (hCG) administration to ovulate a dominant follicle and form an accessory CL has been widely used in an attempt to

improve pregnancy rates, albeit with variable results. These data have been summarized by Lonergan, 2011. In a recent large study Nascimento *et al.* (2013) reported the results of two separate analyses that evaluated the effect of hCG treatment post-AI on fertility in lactating dairy cows. The first study used meta-analysis to combine the results from 10 different published studies that used hCG treatment on day 4 to 9 post-AI in lactating dairy cows. Overall, hCG administration increased pregnancies per artificial insemination (P/AI) by 3.0% (34%, 752/2,213 vs. 37%, 808/2,184). In a subsequent field trial lactating Holstein cows (n = 2,979) from six commercial dairy herds received hCG or not on day 5 after a timed AI; pregnancies per AI were greater in cows treated with hCG (40.8%, 596/1,460) than control (37.3%, 566/1,519) cows. Surprisingly, the positive effect of hCG was restricted to first-lactation cows.

Despite positive effects of administration of exogenous P4 using intravaginal P4 devices on conceptus development (Carter *et al.*, 2008; Clemente *et al.*, 2009) recent studies suggest that this may not translate into improved pregnancy rates (Beltman *et al.*, 2009; Parr *et al.*, School of Agriculture and Food Science, Dublin; unpublished results), possibly due to its potentially negative effects on CL lifespan (O'Hara *et al.*, 2012). More work in this area is required.

### Conclusion

Progesterone is critical for the establishment and maintenance of pregnancy. It has a crucial role in creating an optimal uterine environment in which the embryo can develop, through its actions on the uterine endometrium, and in turn, the composition of the uterine lumen fluid. Strategies aimed at elevating P4 in the early luteal phase have led to variable results in terms of improving pregnancy rates; these variable results may be due to the type of animal treated (nonlactating heifer, lactating dairy cow, beef cow), the endogenous P4 concentrations in such animals and the mode of achieving elevated P4.

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## Uterine diseases in dairy cows: understanding the causes and seeking solutions

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### Abstract

Uterine diseases such as metritis and endometritis are highly prevalent in high producing dairy cows. These diseases lead to impaired welfare and fertility, and result in economic loss. The objective of this review article is to provide the current understanding of the underlying causes of uterine diseases and to provide some strategies to prevent them. The causes of uterine diseases are complex and multifactorial; therefore a holistic approach must be taken when trying identify the causes or prevent them. The dairy cow undergoes a state of negative energy, mineral and vitamin balance during the transition into lactation, which leads to immunosuppression and increased susceptibility to disease. The main risk factors for uterine diseases are primiparity (for metritis only), dystocia, male offspring, twins, stillbirth, abortion, prolapsed uterus, retained placenta (RP), ketosis, and hypocalcemia. Prevention strategies should be focused on maximizing cow comfort and dry matter intake (DMI), preventing hypocalcemia and hyperketonemia, preventing dystocia, prolapsed uterus, abortion, stillbirth and RP. Maximization of cow comfort and DMI can be achieved with appropriate housing and cooling. Management strategies to prevent metabolic and calving related problems include the use of anionic diets, the use of feed additives such as monensin and rumen protected choline, implementation of sound vaccination programs, and the use of sexed semen. Trace mineral and vitamin supplementation beyond what is fed in the diet is still controversial; however some trials have shown a decrease in RP and stillbirths. Prophylactic treatment of cows at high risk for metritis with PGF<sub>2</sub> $\alpha$  and/or oxytocin is not warranted because there is no beneficial effect. Prophylactic treatment of cows at high risk for metritis with NSAIDs is contraindicated because it has been found to decrease DMI and increase the degree of negative energy balance; therefore, leading to an increase in the risk of RP and metritis. Prophylactic treatment of cows at high risk for metritis with estradiol is contraindicated because there is no beneficial effect on the prevention of metritis and there is a negative effect on long term fertility. Prophylactic treatment of cows at high risk for metritis with antibiotics can reduce the incidence of uterine disease but has no positive long term effects on fertility; therefore, decision to implement prophylactic antibiotic treatment should be

based on welfare, economic and legal considerations. Given that most treatments are not very efficacious, efforts should be focused on management strategies to decrease metabolic problems such as hypocalcemia and ketosis, and to prevent risk factors such as dystocia, male calves, abortions, stillbirths, and retained placenta.

**Keywords:** causes, dairy cows, solution, uterine diseases.

### Introduction

Uterine diseases such as metritis and endometritis are highly prevalent in high producing dairy cows. Metritis is characterized by fetid red-brownish uterine discharge within the first 21 days in milk (DIM; Sheldon *et al.*, 2006), and affects about 20.0% of lactating dairy cows, with the incidence ranging from 8 to >40% in some farms (Curtis *et al.*, 1985; Goshen and Shpigel, 2006; Hammon *et al.*, 2006; Huzzey *et al.*, 2007; Galvão *et al.*, 2009b). Clinical endometritis is characterized by the presence of purulent (>50%) uterine discharge after 21 DIM or mucopurulent (50% pus, 50% mucus) after 26 DIM (Sheldon *et al.*, 2006), and also affects about 20.0% of lactating dairy cows, with the prevalence ranging from 5 to >30% in some herds (LeBlanc *et al.*, 2002; McDougall *et al.*, 2007; Galvão *et al.*, 2009b). Subclinical endometritis is defined by the presence of >18% neutrophils (PMN) in uterine cytology samples collected between 21 and 33 DIM or >10% PMN between 34 and 47 DIM (Sheldon *et al.*, 2006), and is the most prevalent of all uterine diseases; it affects approximately 30% of lactating dairy cows, with the prevalence ranging from 11 to >70% in some herds (Kasimanickam *et al.*, 2004; Gilbert *et al.*, 2005; Hammon *et al.*, 2006; Barlund *et al.*, 2008; Galvão *et al.*, 2009a; Cheong *et al.*, 2011). These diseases have been associated with decreased pregnancy per artificial insemination (AI), extended interval to pregnancy, increased culling, and economic losses (Bartlett *et al.*, 1986; Sheldon and Dobson, 2004; Gilbert *et al.*, 2005; Overton and Fetrow, 2008; Galvão *et al.*, 2009a, b).

The decreased fertility is caused by negative effects in the uterus and in the ovary. Uterine diseases cause lesions in the endometrium (Bonnett *et al.*, 1991), disrupt endometrial function (Sheldon and Dobson, 2004), and impair embryo development (Soto *et al.*, 2003; Hill and Gilbert, 2008). Uterine diseases decrease luteinizing

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hormone, first dominant follicle size and growth, and follicular ability to secrete estradiol; therefore affecting ovulatory capacity (Peter *et al.*, 1989; Sheldon *et al.*, 2002; Williams *et al.*, 2008). After postpartum ovulation resumes, cows that developed uterine disease present prolonged luteal phases (Opsomer *et al.*, 2000; Mateus *et al.*, 2002), which can decrease time to insemination and conception rates. In this review, we will focus on understanding the main causes of uterine diseases and present some solutions for the problem.

### Causes

Like most diseases, uterine diseases are multifactorial; therefore it becomes extremely difficult to discuss all factors affecting its occurrence. Some of the traditional risk factors associated with metritis include primiparity, dystocia, male offspring, twins, stillbirth, abortion, prolapsed uterus, retained placenta (RP), ketosis, and hypocalcemia (Erb *et al.*, 1981a, b; Dohoo and Martin, 1984; Markusfeld, 1984, 1985, 1987; Curtis *et al.*, 1985; Gröhn *et al.*, 1990; Correa *et al.*, 1993; Kaneene and Miller, 1995; Goshen and Shpigel, 2006; Dubuc *et al.*, 2010; Ospina *et al.*, 2010; Hosseinzadeh and Ardalan, 2011). Risk factors for endometritis include dystocia, male offspring, twins, stillbirth, abortion, RP, metritis, problems with vulval conformation, and ketosis (Gröhn *et al.*, 1990; Galvão *et al.*, 2009b; Dubuc *et al.*, 2010; Potter *et al.*, 2010; Cheong *et al.*, 2011). Because of the multifactorial nature of uterine diseases, it is helpful to think of the disease triangle (Stevens, 1960) when trying to understand their causes. In that regard, for establishment of disease, it is necessary a susceptible host, a virulent pathogen, and an environment favorable for disease development.

Starting with the host, the dairy cow undergoes dramatic metabolic and physical challenges during the transition to lactation (3 weeks before to 3 weeks after calving). Regarding the metabolic challenge, the transition period is characterized by a state of negative energy, mineral, and vitamin balance (Goff and Horst, 1997) in which there is a decrease in dry-matter intake (DMI), leading to a sharp decrease in glucose, minerals (e.g. calcium, selenium) and vitamins (e.g. A and E) right after parturition, and an increase in body fat mobilization in the form of non-esterified fatty acids (NEFA). High mobilization of NEFA results in excessive uptake by the liver; therefore, leading to incomplete oxidation of this fatty acids and the accumulation of ketone bodies such as beta-hydroxybutyrate (BHBA) in the blood (Vazquez-Añon *et al.*, 1994). This state of negative energy, mineral, and vitamin balance leads to immunosuppression (Kehrli and Goff, 1989; Gilbert *et al.*, 1993; Cai *et al.*, 1994) and increased susceptibility to disease (Trinder *et al.*, 1973; Harrison *et al.*, 1986; Hammon *et al.*, 2006; Galvão *et al.*, 2010, 2011, 2012; Martinez *et al.*, 2012). The metabolic challenge is likely a result of preparation

for and initiation of lactation (Kimura *et al.*, 1999, 2006); however, dairy cows also have a high incidence of dystocia (11 to 29%; Meyer *et al.*, 2001; Schuenemann *et al.*, 2011a) which help breach the physical barriers such as the vulva and endometrium and probably affect DMI intake postpartum because of discomfort. We have observed (Vieira-Neto *et al.*, 2013) that cows that suffer lacerations >2cm have a much greater incidence of metritis than cows with no laceration (63.0 vs. 35.2%;  $P < 0.002$ ), while cows having laceration <2 cm were intermediate (43%). The higher incidence of dystocia in primiparous (29%) probably helps explain the higher incidence of metritis in primiparous cows (Meyer *et al.*, 2001). Stillbirths are also highly correlated with dystocia (Meyer *et al.*, 2001) and hypocalcemia (Martinez *et al.*, 2012). Other risk factors such as twins, prolapsed uterus and RP are correlated among themselves and also associated with hypocalcemia (Risco *et al.*, 1984, 1994; Kimura *et al.*, 2002; Martinez *et al.*, 2012). The RP may also work as a fomite and carry contaminants into the vagina. Abortion is a risk factor probably because of the underlying condition that caused the abortion in the first place and its association with RP.

The dairy cow is unique in the sense that virtually all cows are infected with bacteria in the days following calving (Sheldon and Dobson, 2004). Bacterial culture of the postpartum uterus yields a wide range of isolates (Elliot *et al.*, 1968; Griffin *et al.*, 1974; Sheldon *et al.*, 2002; Galvão *et al.*, 2009b). A complete list of isolates can be found in the work by Williams *et al.* (2005), but mainly *Escherichia coli* (*E. coli*), *Trueperella* (formerly *Arcanobacterium*) *pyogenes* (*T. pyogenes*), *Fusobacterium necrophorum* (*F. necrophorum*), and *Prevotella melaninogenica* (*P. melaninogenica*) were isolated from cows with metritis, whereas *Streptococcus* spp., *Staphylococcus* spp., and *Bacillus* spp. were isolated from healthy cows (Bonnett *et al.*, 1991; BonDurant *et al.*, 1999; Huszenicza *et al.*, 1999; Gilbert *et al.*, 2007). These four main bacteria are believed to work synergistically to cause uterine disease in dairy cows (Griffin *et al.*, 1974; Ruder *et al.*, 1981; Bonnett *et al.*, 1991). In fact, *E. coli* increases the susceptibility of the endometrium to subsequent infection with *T. pyogenes* (Olson *et al.*, 1984; Gilbert *et al.*, 2007; Williams *et al.*, 2007), and *T. pyogenes* acts synergistically with *F. necrophorum* and *P. melaninogenica* to enhance the severity of uterine disease (Griffin *et al.*, 1974; Ruder *et al.*, 1981; Bonnett *et al.*, 1991). Recent work has highlighted the importance of *E. coli* on the development of metritis and endometritis (Bicalho *et al.*, 2010, 2012; Sheldon *et al.*, 2010; Machado *et al.*, 2012a, b); especially the fact that it predisposes to infection with other pathogenic bacterium such as *F. necrophorum* and *T. pyogenes* (Bicalho *et al.*, 2012; Machado *et al.*, 2012a, b), increases the likelihood of developing metritis and endometritis, and decreases the likelihood of conception (Bicalho *et al.*, 2010, 2012; Machado *et al.*, 2012a).

Very few studies have tried to evaluate the



effect of the environment on the incidence of bacterial contamination of the uterus or the incidence of uterine disease. Noakes *et al.* (1991) compared the bacterial flora of the uterus from 26 cows from two herds with contrasting hygiene environments (one with poor hygiene and one with good hygiene), and found similar proportion of cows with uterine contamination and similar proportion of the main uterine pathogens. Based on these findings, the authors concluded that the environment had no influence on either the quantitative or qualitative uterine bacterial flora; therefore, uterine disease was due to other factors. This was a small and uncontrolled study; therefore the findings should be interpreted carefully. A larger study (Cheong *et al.*, 2011) with 38 herds from upstate New York looked at the effect of bedding material in the calving pen and type of housing early postpartum. They found that herds that used straw in the calving pens had 10.7% ( $P < 0.005$ ) lower incidence of subclinical endometritis compared to other types of bedding (sand, sawdust or paper). They also found that herds that housed their fresh cows in free-stalls had 16.7% (36.1 vs. 19.4%;  $P < 0.005$ ) lower incidence of subclinical endometritis than herds that housed their cows in bedded packs. Although the results were significant, for type of bedding at the calving pen and type of housing early postpartum, a direct link between hygiene in the environment and incidence of disease could not be made; therefore, the authors warned the readers to interpret the results with caution. Although environment hygiene has not been associated with incidence of uterine disease, perineal hygiene at the time of calving has. In a study with 562 cows in Ohio (Schuenemann *et al.*, 2011b), the hygiene of perineum of cows right before calving was scored using a 1-3 scale (1 = free of dirt-manure and completely dry; 2 = slightly wet, dirt-manure in 1-10% of the surface; 3 = moderately

wet, covered with dirt-manure in >10% of the surface). Cows with scores 3 or 2 had greater incidence of metritis ( $22.4 \pm 6\%$  and  $18.9 \pm 4\%$ , respectively) than cows with a score 1 ( $10.8 \pm 3\%$ ;  $P < 0.05$ ). These results indicate that contamination of the uterus might be coming from the cow herself and not from the environment.

An interesting observation is the difference in incidence of uterine disease in cows on free-stalls and cows on pasture. Certainly, there are many differences between the two types of cows besides the type of housing (milk yield and genotype being important ones). However, data recently generated in Florida (Ribeiro *et al.*, 2013) shows that Holstein cows on pasture have much lower incidence of metritis (4.3 vs. 16.1%) and clinical endometritis (11.7 vs. 20.8%) than what is seen for cows in free-stalls (Santos *et al.*, 2010), while other diseases such as mastitis (22.0 vs. 12.2%), pneumonia (2.4 vs. 2.0), and indigestion (3.9 vs. 2.8%) seem unaffected. In the study by Ribeiro *et al.* (2013), the authors speculated that the low incidence of uterine disease, especially metritis was due to the low incidence (8.5%) of calving problems (dystocia, twins, stillbirth or RP), which may be related to smaller calf size since most Holstein cows were bred with Jersey sires. This highlights the importance of calf size and consequently calving ease as a risk factor for uterine disease. One study pointed out that a male offspring, which is larger, had the highest influence (as measured by the population attributable fraction) in the incidence of endometritis (Potter *et al.*, 2010). Mee (2012) summarized data on dystocia from several countries from 2000 to 2011 (Table 1) and found large differences in dystocia incidence in the Holstein-Friesian population of cows from different countries (the USA being the highest) and among different breeds of dairy cows; therefore, there is potential for reduction of dystocia incidence through genetic selection.

Table 1. International prevalence of dystocia in dairy heifers and cows (2000-2011).

Country	Breed of dam	Heifers, %	Heifers & Cows, %	Reference
Australia	Holstein-Friesian	9.5	4.1	McClintock, 2004
Canada	Holstein-Friesian	NR <sup>a</sup>	6.9	Sewalem <i>et al.</i> , 2008
Denmark	Holstein-Friesian	8.7	NR	Hansen <i>et al.</i> , 2004
Ireland	Holstein-Friesian	9.3	6.8	Mee <i>et al.</i> , 2011
France	Holstein-Friesian & Normande	NR	6.6	Fourichon <i>et al.</i> , 2001
New Zealand	Holstein-Friesian	6.5	3.8	Xu and Burton, 2003
Norway	Norwegian Red	2.7	1.1	Heringstad <i>et al.</i> , 2007
Spain	Holstein-Friesian	3.1	2.5	Lopez de Maturana <i>et al.</i> , 2006
Sweden	Swedish Red and White	3.9	1.9 <sup>b</sup>	Steinbock, 2006
The Netherlands	Holstein-Friesian	NR	7.8 <sup>c</sup>	Eaglen and Bijma, 2009
UK	Holstein-Friesian	6.9	2.0 <sup>b</sup>	Rumph and Faust, 2006
USA	Holstein-Friesian	22.6	13.7	Gevrekci <i>et al.</i> , 2006

<sup>a</sup>Not recorded, <sup>b</sup>Cows only, <sup>c</sup>Second calvers only. Adapted from Mee (2012).



### Potential solutions

Although complete elimination of uterine disease does not seem possible with our current understanding of the pathophysiology of uterine diseases, there are management strategies that can be taken to mitigate the problem. Prevention strategies should be focused on maximizing cow comfort and DMI, preventing late term abortions with appropriate vaccination programs, favoring the birth of female calves with the use of sexed semen, preventing hypocalcemia and hyperketonemia, and preventing mineral and vitamin deficiencies. Prophylactic treatment with prostaglandin F<sub>2</sub>-alpha (PGF<sub>2</sub>α), oxytocin, estradiol, nonsteroidal anti-inflammatory drugs (NSAIDs), and antibiotics will also be discussed. Treatment of uterine diseases will not be discussed because it has been covered in previous publications (Galvão, 2012).

Dry matter intake is the single most critical factor of dairy production, and its effects on uterine health have been clearly demonstrated. Cows that develop metritis and endometritis have decreased dry matter intake starting up to two weeks before calving and remaining until four to five weeks after calving (Hammon *et al.*, 2006; Huzzey *et al.*, 2007). Critical areas of facility design related to cow comfort and DMI include access to feed and water, stall design and surface, supplemental lighting, ventilation, and cow cooling. A nice review of all these factors was put together by researcher at Kansas State University (Brouk and Smith, 2000). They emphasize that careful consideration must be made when designing facilities due to the fact that once they are built they will affect the performance of animals for the life of the facility (>20 years).

Maintenance of calcium homeostasis throughout transition is imperative for uterine health (Goff and Horst, 1997; Martinez *et al.*, 2012). The use of anionic salts can reduce the incidence of clinical hypocalcemia (milk fever) to <2% in multiparous cows and also reduce the incidence of subclinical hypocalcemia in early postpartum (Horst *et al.*, 1997). However, anionic salts must be used with caution because they may reduce dry matter intake, especially if >300 meq of anions/kg are fed (Charbonneau *et al.*, 2006). They should also only be fed to close-up (usually 3 weeks before calving) dry cows. There is a debate to whether nulliparous cows should receive anionic salts (Horst *et al.*, 1997) because of a potential decrease in DMI (Moore *et al.*, 2000); however, with the availability of more palatable salts, feeding nulliparous cows should not be a problem (DeGroot *et al.*, 2010). In order to achieve success using anionic salts, controlled feeding, precise ration formulation using the dietary cation-anion difference (DCAD) concept, and monitoring of urine pH are necessary. The goal is to have urine pH between 6 and 7. This can usually be

achieved with a DCAD between -5 and -15 milliequivalents per 100 g of dry matter (Horst *et al.*, 1997). Nevertheless, even with the use of anionic salts, between 20 and 50% of postpartum cows will be hypocalcemic (serum total Ca concentrations <8.5 mg/dl) early postpartum, and these cows will have a much higher incidence of metritis than normocalcemic cows (Martinez *et al.*, 2012). In the same work (Martinez *et al.*, 2012), it was observed that cows with dystocia, twins, stillbirth and RP had a greater decrease in calcium postpartum than cows without these risk factors; therefore, if an effective postpartum treatment was available, it would probably benefit this group of animals. The problem is that to this date, no effective treatment is available. Benzaquen *et al.* (2008) treated cows with dystocia with calcium propionate at 516 g of calcium propionate (providing 110 g of calcium and 400 g of propionate, 1.5 g of zinc, and 0.5 g of copper) at 6 and 72 h postpartum and actually found that calcium propionate treatment prevented the physiological increase in calcium concentration; therefore, resulting in lower calcium concentration on days two and three postpartum.

Trace mineral and vitamin deficiency early postpartum, particularly selenium and vitamin E have long been identified as a cause of uterine disease (Trinder *et al.*, 1973; Harrison *et al.*, 1986), probably because of the effect on neutrophil function (Cebra *et al.*, 2003). Although selenium supplementation is recommended, the Federal Drug Administration (FDA) limits the supplementation of selenium to 0.3 ppm (mg/kg); therefore, because the upper limit of supplementation is set, the only options to try to supplement more is to change the source of selenium. Organic selenium (selenium yeast) is more absorbable than inorganic selenium (selenite and selenate). One study in Florida (Silvestre *et al.*, 2006) and one in California (Rutigliano *et al.*, 2008) compared the two sources of selenium. Only the study in Florida observed a decrease in clinical endometritis and an increase in conception rate to second service (Silvestre *et al.*, 2006); nonetheless, neither study found any positive impact in the first service conception rate. Out of the two studies, blood concentrations of selenium were only increased in the study in Florida. The authors from the study in California pointed out that Selenium concentration in forages were quite high, which probably masked any benefits from selenium yeast. Therefore, it is important to know the selenium status of the ration as a whole before making a decision to adopt the supplementation of selenium yeast.

There is vast literature on the effect of vitamin E on milk quality (somatic cell count) and mastitis incidence; however, the evidence for an effect on uterine health and fertility is limited. Supplementation with 3000 IU vitamin E/cow/day in the late dry period is recommended because it is generally associated with decreased risk of mastitis postpartum (Politis, 2012).



Few studies have looked at the effect of vitamin E supplementation, beyond what is provided in the feed, on uterine health. In a review of the available literature, Allison and Laven (2000) stated that there appeared to be little benefit of high levels of vitamin E (at least 1000 iu per day) supplementation during the dry period on infectious diseases other than mastitis. They said that in herds with a history of selenium deficiency and a high incidence of RP, supplementation of vitamin E, in conjunction with selenium, could reduce RP, but the evidence for an effect of supplementation on other reproductive diseases was limited. In one study where 2100 mg of vitamin E and 7 g of sodium selenite were supplemented by intramuscular administration 2 weeks before calving and on the day of calving, there was a tendency ( $P = 0.055$ ) for reduced incidence of RP, but there was no effect on time to conception (Bourne *et al.*, 2008). In another study where daily supplementation with 1,610 mg of RRR- $\alpha$ -tocopherol (vitamin E) was performed from 4 weeks before to 2 weeks after calving, a reduction in the proportion of stillbirths was observed, but again no effect on long term fertility was observed (Persson *et al.*, 2007).

Supplementation with injectable trace minerals has produced controversial results. Studies have found that additional supplementation of trace minerals can have a negative (Vanegas *et al.*, 2004), positive (Sales *et al.*, 2011), or neutral effect on reproductive performance (Vanegas *et al.*, 2004). Vanegas *et al.* (2004) observed that trace mineral supplementation (Cu, Mn, and Zn) postpartum did not affect cow reproductive performance; however, supplementation pre and postpartum decreased reproductive performance. On the other hand, a recent study observed that trace mineral supplementation (Cu, Mn, Zn, and Se) before and after calving reduced the incidence of stillbirth and endometritis; nonetheless, it did not affect RP, metritis, or long term fertility (Machado *et al.*, 2013). In a study performed with crossbred heifers, there was an increase in the conception rate (embryo survival) of heifers that received a trace mineral supplementation (Cu, Mn, Zn, and Se) 17 days prior to embryo transfer (Sales *et al.*, 2011). An excellent meta-analysis was performed by Rabiee *et al.* (2010) on the effects of feeding organic trace minerals (OTM) on milk yield and reproductive performance in lactating dairy cows. They observed that feeding organic trace minerals significantly increased milk production by 0.93 kg/day, milk fat yield by 0.04 kg/day, and milk protein yield by 0.03 kg/day. However, the response to supplementation with OTM was not consistent across trial. Meta-regression analysis showed that milk production increased with the use of other supplements (e.g., monensin) and for 4-Plex versus Availa-4. Feeding OTM before calving and feeding for a full lactation after calving also increased milk production. Supplementation of cows with OTM reduced days open by 13.5 days and the number of services per conception by 0.27 units. The risk of

pregnancy by 150 days in lactation was greater in cows fed OTM, but OTM had no significant effect on the interval from calving to first service or on the 21-day pregnancy rate. Although supplementation of vitamins and trace mineral remain controversial, there is a mounting body of evidence pointing to its beneficial effect.

Because of the importance of energy balance on the incidence of uterine disease, nutritional supplements that prevent ketosis may be an important component for the prevention of uterine diseases. Two nutritional supplements, monensin, and choline have shown consistent results on the improvement of energy balance and fat metabolism, respectively (Zahra *et al.*, 2006). Monensin has been shown to increase glucose concentrations postpartum, and both monensin and choline have been shown to decrease ketosis postpartum; therefore, they are expected to have a positive impact on uterine health (Zahra *et al.*, 2006; Lima *et al.*, 2012). In one study (Lima *et al.*, 2012), feeding rumen protected choline pre and postpartum reduced the incidence of clinical ketosis, and mastitis; however, it did not influence cyclicity or conception rates. Feeding rumen protected choline postpartum only, had mixed results.

Administration of a wide range of drugs (PGF2 $\alpha$ , oxytocin, estradiol, NSAIDs, antibiotics) is commonly performed in dairy cows, particularly in cows with RP or dystocia, in an attempt to prevent uterine diseases. The benefits of such use are controversial. Because the mechanism of release of the placenta mainly involves the action of leukocytes and collagenase, the use of PGF2 $\alpha$  or oxytocin are not expected to be helpful (Beagley *et al.*, 2010). In two very nice reviews of the literature (Frazer, 2005; Beagley *et al.*, 2010), both authors did not recommend the use of either PGF2 $\alpha$  or oxytocin for prevention or treatment of RP because these hormones are not major players in the release of the placenta and they are already increased in cows with RP. Nonetheless, some studies have observed a reduction in the incidence of RP when either PGF2 $\alpha$  (Stocker *et al.*, 1993) or oxytocin (Mollo *et al.*, 1997) are used; however, several other studies have found no effect (Garcia *et al.*, 1992; Stevens and Dinsmore, 1997; Drillich *et al.*, 2005; Palomares *et al.*, 2010). In both reviews (Frazer, 2005; Beagley *et al.*, 2010), it is recommended that manual removal should not be attempted because it decreases uterine defense mechanisms (Paisley *et al.*, 1986; Peters and Laven, 1996) and impairs subsequent fertility (Bolinder *et al.*, 1988). Nonsteroidal anti-inflammatory drugs are also commonly used in the attempt to prevent uterine diseases; however, counter intuitively, its use has caused a decrease in DMI and an increase in the degree of negative energy balance; therefore, leading to an increase in the risk of RP and metritis (Waelchli *et al.*, 1999; Duffield, *et al.*, 2009; Shwartz *et al.*, 2009). For this reason, the prophylactic use of NSAIDs is not



recommended. Estradiol has also been used for prevention of metritis in cows with RP (Risco and Hernandez, 2003) and other risk factors such as dystocia stillbirth and twins (Overton *et al.*, 2003); however, its use is not recommended because it does not prevent metritis (Risco and Hernandez, 2003; Overton *et al.*, 2003) and is detrimental to fertility (Risco and Hernandez, 2003). The only known drug shown to release the placenta is collagenase. Administration of 20,000-200,000 U of bacterial collagenase into the umbilical artery has been shown to prevent RP or to hasten release of the placenta in several studies (Eiler and Hopkins, 1993; Eiler *et al.*, 1997; Guerin *et al.*, 2004); however, long term effects of uterine health or fertility have not been carried out. The most consistent results for prevention of metritis have been the treatment of cows with RP with antibiotics. Several studies have shown that the incidence of metritis (Overton *et al.*, 2003; Risco and Hernandez, 2003; McLaughlin *et al.*, 2013) or endometritis (Dubuc *et al.*, 2011) can be decreased with systemic antibiotic administration; however, some studies have found no effect on the prevention of metritis (Drillich *et al.*, 2006; Dubuc *et al.*, 2012) and no study have found a positive impact of treatment of cows with RP on long term fertility, culling, or milk production (Overton *et al.*, 2003; Risco and Hernandez, 2003; Goshen and Shpigel, 2006; Dubuc *et al.*, 2011; McLaughlin *et al.*, 2013). Furthermore, in the USA, the FDA has banned the use of ceftiofur (the only molecule with no milk withdrawal) for preventative treatment. Therefore, prophylactic antibiotic treatment should be based on welfare, economical, and legal considerations.

### Conclusions

Given that most treatments are not very efficacious, efforts should be focused on management strategies to decrease metabolic problems such as hypocalcemia and ketosis, and to prevent risk factors such as dystocia, male calves, abortions, stillbirths, and RP.

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## Biological roles of progesterone, prostaglandins, and interferon tau in endometrial function and conceptus elongation in ruminants

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### Abstract

A large majority of pregnancy loss in cattle occurs during the first three weeks after conception, particularly during the peri-implantation period. This review integrates established and new information on the biological role of ovarian progesterone (P4), prostaglandins (PGs) and interferon tau (IFNT) in endometrial function and conceptus elongation during the peri-implantation period of pregnancy in ruminants. Progesterone is secreted by the ovarian corpus luteum (CL) and is the unequivocal hormone of pregnancy. Prostaglandins are produced from both the endometrium as well as conceptus trophoderm during early pregnancy. Interferon tau is produced solely by the conceptus trophoderm and is the maternal recognition of pregnancy signal that inhibits production of luteolytic pulses of PGF<sub>2α</sub> by the endometrium to maintain the CL and thus production of P4. Conceptus-endometrial interactions in ruminants are complex and involve carefully orchestrated temporal and spatial alterations in endometrial gene expression during pregnancy. Available results support the idea that the individual, interactive, and coordinated actions of P4, PGs, and IFNT regulate uterine receptivity to conceptus implantation by controlling expression of genes in the endometrium and that their actions are essential for conceptus elongation. One outcome of gene expression changes in the endometrial epithelia is alterations in luminal secretions that govern conceptus elongation via effects on the trophoderm. An increased knowledge of conceptus-endometrial interactions during early pregnancy in ruminants is necessary to understand and elucidate the causes of infertility and recurrent pregnancy loss and to provide a basis for new strategies to improve fertility, pregnancy outcomes and thus reproductive efficiency.

**Keywords:** conceptus, endometrium, interferon, pregnancy, prostaglandin, ruminant.

### Introduction

This review integrates established and new information on the biological role of ovarian progesterone (P4), prostaglandins (PGs) and interferon tau (IFNT) in endometrial function and conceptus elongation during the peri-implantation period of pregnancy in ruminants. This area of reproduction is

particularly important due to relatively high levels of pregnancy loss. In cattle, estimates indicate that fertilization rate is 90% with an average calving rate of about 55%, indicating an embryonic-fetal mortality of about 35% (Diskin *et al.*, 2006). Further, 70 to 80% of total embryonic loss occurs during the first 3 weeks after insemination (Diskin *et al.*, 2006; Diskin and Morris, 2008), particularly between days 7 and 16 (Diskin and Sreenan, 1980; Roche *et al.*, 1981; Berg *et al.*, 2010). Embryo mortality is greater in non-lactating cows than heifers (Berg *et al.*, 2010), and early pregnancy loss is even greater in high producing lactating dairy cattle and can approach 70% (Evans and Walsh, 2011; Thatcher *et al.*, 2011). Infertility and subfertility are also major cost factors in the cattle embryo transfer (ET) industry (Looney *et al.*, 2006). Mean survival rate to calving following transfer of *in vivo*-derived embryos from superovulated donors is only 43% with a range from 31 to 60% (McMillan, 1998), whereas the mean survival rate after transfer of *in vitro*-produced embryos is lower and ranges from 30 to 40% (McMillan, 1998; Hansen and Block, 2004). Although embryonic mortality is certainly a problem in cattle, our knowledge of the complex biological and genetic mechanisms governing endometrial receptivity and conceptus elongation and implantation is limited in domestic ruminants (Ulbrich *et al.*, 2013).

Establishment of pregnancy in domestic ruminants (i.e., sheep, cattle, goats) begins at the conceptus stage and includes pregnancy recognition signaling, implantation, and placentation (Guillomot, 1995; Spencer *et al.*, 2004, 2007a, 2008). The morula-stage embryo enters the uterus on days 4 to 6 post-mating and then forms a blastocyst that contains an inner cell mass and a blastocoele or central cavity surrounded by a monolayer of trophoderm. After hatching from the zona pellucida (days 8 to 10), the blastocyst slowly grows into a tubular or ovoid form and is then termed a conceptus (embryo-fetus and associated extraembryonic membranes; Guillomot, 1995; Hue *et al.*, 2012). In sheep, the ovoid conceptus of about 1 cm on day 11 begins to elongate on day 12 and forms a filamentous conceptus of 10 to 15 cm or more in length that occupies the entire length of the uterine horn ipsilateral to the corpus luteum (CL). In cattle, the hatched blastocyst forms an ovoid conceptus between days 12 to 14 and is only about 2 mm in length on day 13. By day 14, the conceptus is about 6 mm, and

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the elongating bovine conceptus reaches a length of about 60 mm (6 cm) by day 16 and is 20 cm or more by day 19. Indeed, the bovine blastocyst/conceptus doubles in length every day between days 9 and 16 with a significant increase (~10-fold) in growth between days 12 and 15 (Betteridge *et al.*, 1980; Berg *et al.*, 2010). After day 16 in sheep and day 19 in cattle, the elongating conceptus begins the process of implantation and placentation (Guillomot *et al.*, 1981). Conceptus elongation involves exponential increases in length and weight of the trophoblast (Wales and Cuneo, 1989) and onset of extraembryonic membrane differentiation, including gastrulation of the embryo and formation of the yolk sac and allantois that are vital for embryonic survival and formation of a functional placenta (Guillomot, 1995; Hue *et al.*, 2012). Trophoblast elongation observed in ruminants is not due to the geometrical change of cell shape but is likely the consequence of cell addition associated with peculiar plans of cell division or intercalation (Wang *et al.*, 2009).

Blastocyst growth into an elongated conceptus does not occur *in vitro*, as it is dependent on ovarian P4 and secretions supplied by the endometrium of the uterus (Betteridge and Flechon, 1988; Gray *et al.*, 2001b; Lonergan, 2011). The trophoblast of the elongating conceptus synthesizes and secretes prostaglandins (PGs) and interferon tau (IFNT) in ruminants (Lewis, 1989; Ulbrich *et al.*, 2009; Forde and Lonergan, 2012; Dorniak *et al.*, 2013b). Interferon tau is the signal for maternal recognition of pregnancy in ruminants and is secreted predominantly by the elongating conceptus (Roberts *et al.*, 2003; Robinson *et al.*, 2006). As a pregnancy recognition signal, IFNT ensures continued production of P4 by the CL (Thatcher *et al.*, 1989; Spencer *et al.*, 2007a). Additionally, IFNT stimulates transcription of a number of genes and activities of several enzymes in a cell-specific manner within the endometrium implicated in establishment of uterine receptivity and conceptus elongation and implantation (Hansen *et al.*, 1999; Spencer *et al.*, 2007a; Dorniak *et al.*, 2013a). The endometrium itself, as well as the ovoid and elongating conceptuses, produces PGs during early pregnancy (Marcus, 1981; Lewis, 1989). The precise role of conceptus-derived PGs remains to be determined in cattle (Ulbrich *et al.*, 2009); however, PGs regulate conceptus growth and elongation in sheep through modulation of endometrial genes important for elongation of the conceptus (Dorniak *et al.*, 2011, 2012b).

The endometrium of the uterus secretes substances, collectively termed histotroph, that govern elongation of the conceptus via effects on trophoblast proliferation and migration as well as attachment and adhesion to the endometrial luminal epithelium (LE; Spencer *et al.*, 2007b, 2008; Bazer *et al.*, 2010). Histotroph is derived primarily from transport and/or synthesis and secretion of substances by the endometrial LE and glandular epithelia (GE), and it is a complex and rather undefined mixture of proteins,

lipids, amino acids, sugars, and ions (Bazer, 1975; Gray *et al.*, 2001a; Koch *et al.*, 2010; Bazer *et al.*, 2012). The recurrent early pregnancy loss observed in uterine gland knockout (UGKO) ewes established the importance of uterine epithelial-derived histotroph for support of conceptus elongation and implantation (Gray *et al.*, 2001b). Available evidence supports the idea that ovarian P4 induces expression of a number of genes, specifically in the endometrial epithelia, that are then further stimulated by factors from the conceptus (e.g., IFNT and PGs) as well as the endometrium itself (e.g., PGs; Dorniak *et al.*, 2013a). The genes and functions regulated by these hormones and factors in the endometrial epithelia elicit specific changes in the intrauterine histotrophic milieu necessary for conceptus elongation (Spencer *et al.*, 2007b, 2008; Bazer *et al.*, 2010; Forde and Lonergan, 2012; Dorniak *et al.*, 2013a).

### Progesterone regulation of endometrial function and conceptus elongation

Progesterone stimulates and maintains endometrial functions necessary for conceptus growth, implantation, placentation, and development to term. In cattle, concentrations of P4 in early pregnancy clearly affect embryonic survival during early pregnancy (Mann and Lamming, 2001; Lonergan, 2011). In both lactating dairy cows and heifers, there is a strong positive association between the post-ovulatory rise in P4 and embryonic development. Increasing concentrations of P4 after ovulation enhanced conceptus elongation in beef heifers (Garrett *et al.*, 1988; Carter *et al.*, 2008), dairy cows (Mann *et al.*, 2006), and sheep (Satterfield *et al.*, 2006), while lower P4 concentrations in the early luteal phase retarded embryonic development in sheep and cattle (Nephew *et al.*, 1991; Mann and Lamming, 2001; Forde *et al.*, 2011a). Supplementation of cattle with P4 during early pregnancy has equivocal effects to increase embryonic survival (Beltman *et al.*, 2009a). However, P4 supplementation is unlikely to rescue development of embryos with inherent genetic defects or in high-producing dairy cows (Mann *et al.*, 2006; Lonergan *et al.*, 2007; Wiltbank *et al.*, 2011).

Progesterone predominantly exerts an indirect effect on the conceptus via the endometrium to regulate blastocyst growth and conceptus elongation (Clemente *et al.*, 2009; Forde *et al.*, 2011a; Larson *et al.*, 2011). Similar to the human (Giudice and Ferenczy, 1996; Kao *et al.*, 2002), endometria of both cyclic and pregnant sheep and cattle express genes implicated in uterine receptivity, which can be defined as a physiological state of the uterus when conceptus growth and implantation for establishment of pregnancy is possible. The absence of a sufficiently developed conceptus to signal pregnancy recognition results in those genes being 'turned off' as luteolysis ensues and the animal returns to estrus for another opportunity to mate. The outcome of the P4-induced changes in the cyclic and pregnant uterus



is to modify the intrauterine milieu, such as an increase in select amino acids, glucose, cytokines, and growth factors in histotroph, for support of blastocyst growth into an ovoid conceptus and elongation to form a filamentous conceptus (Spencer *et al.*, 2008; Bazer *et al.*, 2010; Forde and Lonergan, 2012).

*Sheep*

Actions of ovarian P4 on the uterus are essential for conceptus survival and growth in sheep (Satterfield *et al.*, 2006). Between days 10 and 12 after onset of estrus or mating in cyclic and pregnant ewes (Fig. 1 and Table 1), P4 induces the expression of many conceptus elongation- and implantation-related genes. The initiation of expression of those genes requires P4 action and is temporally associated with the loss of progesterone receptors (PGR) between days 10 and 12 in the endometrial LE and between days 12 and 14 to 16 in the GE after onset of estrus; however, PGR expression is not lost in the stroma or myometrium in the ovine uterus (Spencer and Bazer, 2002). In the

endometrial LE and superficial GE (sGE), P4 induces genes that encode secreted attachment and migration factors (galectin-15 [LGALS15], IGFBP1), intracellular enzymes (prostaglandin G/H synthase and cyclooxygenase 2 [PTGS2] and hydroxysteroid (11-beta) dehydrogenase 1 [HSD11B1]), secreted proteases (cathepsin L [CTSL]), secreted protease inhibitors (cystatin C [CST]3 and 6), a secreted cell proliferation factor (gastrin releasing peptide [GRP]), glucose transporters (SLC2A1, SLC2A5, SLC5A1), and a cationic amino acid (arginine, lysine, and ornithine) transporter (SLC7A2; Spencer *et al.*, 2007a, 2008; Bazer *et al.*, 2010). In the endometrial GE, P4 induces genes that encode for a secreted cell proliferation factor (GRP), a glucose transporter (SLC5A11), secreted adhesion protein (secreted phosphoprotein one or SPP1), a regulator of calcium/phosphate homeostasis (stanniocalcin one or STC1), and an immunomodulatory factor (SERPINA14, also known as uterine milk proteins or uterine serpins). Several of the P4-induced epithelia genes are further stimulated by the actions of PGs and/or IFNT.

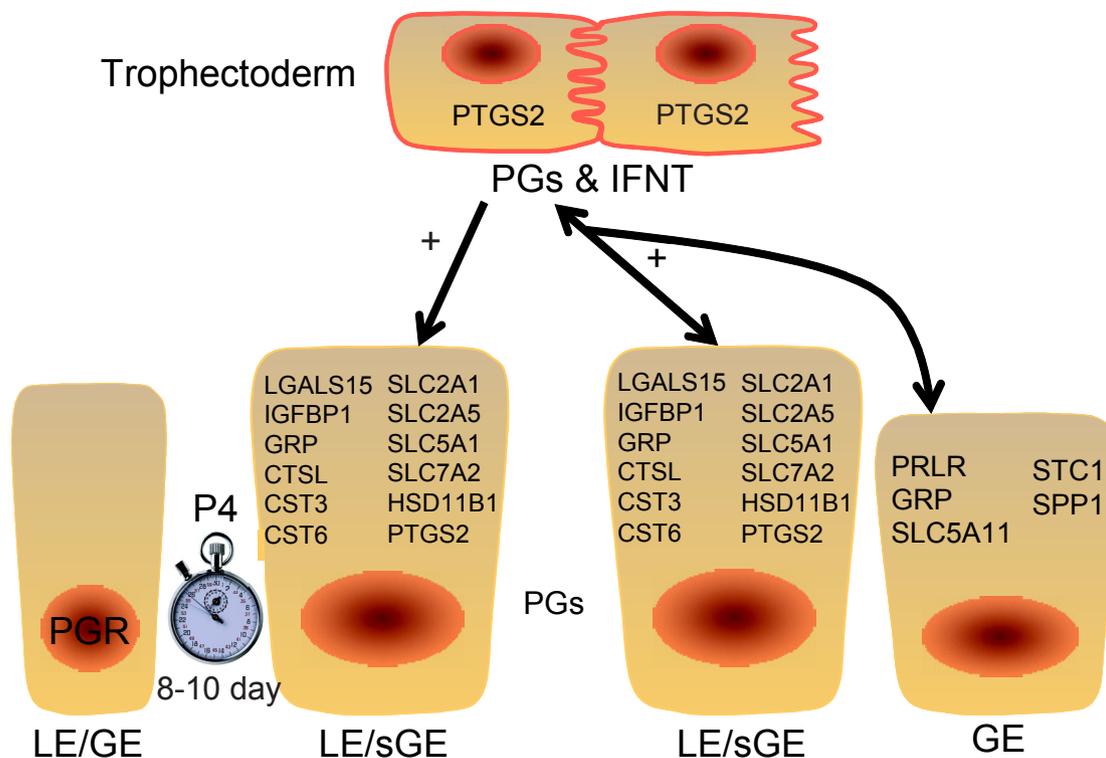


Figure 1. Schematic illustrating the effects of ovarian hormones and factors from the endometrium and conceptus trophoblast on expression of elongation- and implantation-related genes in the endometrial epithelia of the ovine uterus during early pregnancy. Progesterone action for 8-10 days down-regulate expression of the progesterone receptor (PGR). The loss of PGR is correlated with the induction of many genes in the endometrial LE and sGE, including PTGS2 involved in prostaglandin (PG) production in both cyclic and pregnant ewes. If the ewe is pregnant, the trophoblast synthesizes and secretes PGs and interferon tau (IFNT) that act on the endometrium in a cell-specific manner to up-regulate the expression of many P4-induced genes that govern endometrial functions and/or elongation of the conceptus. Legend: GE, glandular epithelia; IFNT, interferon tau; LE, luminal epithelium; PG, prostaglandins; PGR, progesterone receptor; sGE, superficial glandular epithelia. Adapted from Dorniak *et al.* (2012).

Table 1. Effects of ovarian progesterone and intrauterine infusions of interferon tau (IFNT) or prostaglandins (PGs) on elongation- and implantation-related genes expressed in the endometrial epithelia of the ovine uterus<sup>1</sup>

Gene symbol	Progesterone	IFNT	PGs <sup>2</sup>
Transport of glucose			
<i>SLC2A1</i>	↑	+	+
<i>SLC2A5</i>	n.d.	n.e.	+
<i>SLC2A12</i>	n.d.	+	n.e. or +
<i>SLC5A1</i>	↑	+	n.e. or +
<i>SLC5A11</i>	↑	+	n.e. or +
Transport of amino acids			
<i>SLC1A5</i>	n.d.	n.d.	+
<i>SLC7A2</i>	↑	+	n.e.
Cell proliferation, migration and(or) attachment			
<i>GRP</i>	↑	+	+
<i>IGFBP1</i>	↑	+	++
<i>LGALS15</i>	↑	++	++
<i>SPP1</i>	↑	+	n.d.
Proteases and their inhibitors			
<i>CTSL</i>	↑	++	n.e. or +
<i>CST3</i>	↑	+	n.e. or +
<i>CST6</i>		+	n.e.
Enzymes			
<i>HSD11B1</i>	↑	+	++
<i>PTGS2</i>	↑	n.e. (+ activity)	n.e. (+ activity)
Transcription factors			
<i>HIF1A</i>	↑	+	n.e. or +
<i>HIF2A</i>	↑	+	n.e. or +

<sup>1</sup>Effect of hormone or factor denoted as induction (↑), stimulation (+), no effect (n.e.), decrease (-) or not determined (n.d.). <sup>2</sup>Summary data for infusion of PGE<sub>2</sub>, PGF<sub>2α</sub>, or PGI<sub>2</sub> (Dorniak *et al.*, 2012).

### Cattle

Comparisons of the endometrial transcriptome in cyclic and pregnant heifers (days 5, 7, 12, and 13) found no difference prior to pregnancy recognition (days 15 or 16; Forde *et al.*, 2011b; Bauersachs *et al.*, 2012). Indeed, the major changes required to drive conceptus elongation and establish uterine receptivity to implantation occur between days 7 and 13 in response to ovarian P<sub>4</sub>, irrespective of whether an appropriately developed embryo/conceptus is present or not (Forde *et al.*, 2009, 2010, 2011a, b, 2012b; Simmons *et al.*, 2009; Forde and Lonergan, 2012). Similar to sheep, PGR protein is lost from the LE by day 13 and in the GE by day 16, and PGR loss is associated with the down- and up-regulation of genes expressed in the endometrial epithelia (Okumu *et al.*, 2010). Using a global gene profiling approach, studies have identified the temporal changes that occur in endometrial gene expression in both cyclic (Forde *et al.*, 2011a) and pregnant (Forde *et al.*, 2009) heifers following an elevation or diminution of post-ovulatory P<sub>4</sub> during metestrus that promotes or delays conceptus elongation, respectively (Beltman *et al.*, 2009b; Clemente *et al.*, 2009; Forde *et al.*, 2011a). As summarized in a recent review by Forde and Lonergan (Forde and Lonergan, 2012), the expression of several genes are lost in the LE and GE, including

PGR and a protease (alanyl (membrane) aminopeptidase [ANPEP]), and in the GE, including a lipase (lipoprotein lipase [LPL]), protease (matrix metalloproteinase 2 [MMP2]) and immunomodulatory protein with antimicrobial activity (lactotransferrin [LTF]) between days 7 and 13 after onset of estrus or mating in cyclic and pregnant heifers. As expected, many conceptus elongation- and implantation-related genes appear in the endometrial epithelia between days 7 and 13 in cyclic and pregnant heifers. Genes up-regulated in the LE encode a mitogen (connective tissue growth factor [CTGF]) and in the GE encode a transport protein (retinol binding protein 4 [RBP4]), a glucose transporter (SLC5A1), and a protein involved in transport and cell proliferation (fatty acid binding protein 3 [FABP3]). Further, some genes are up-regulated in both the LE and GE that encode secreted attachment and migration factors (lectin, galactoside-binding, soluble, 9 [LGALS9] and IGFBP1) as well as an intracellular enzyme (PTGS2). It is quite clear that substantial differences in gene expression occur between the receptive endometrium of sheep and cattle, as one of the most abundant genes (LGALS15) induced by P<sub>4</sub> and stimulated by IFNT in the endometrium of sheep is not expressed in cattle (Lewis *et al.*, 2007). However, PTGS2 and IGFBP1 are common endometrial receptivity markers and regulators of conceptus



elongation in both sheep and cattle (Simmons *et al.*, 2009; Dorniak *et al.*, 2012a).

### Interferon tau regulation of endometrial function and conceptus elongation

Maternal recognition of pregnancy is the physiological process whereby the conceptus signals its presence to the maternal system and prolongs the lifespan of the ovarian CL (Bazer *et al.*, 1991). In ruminants, IFNT is the pregnancy recognition signal secreted by the elongating conceptus that acts on the endometrium to inhibit development of the luteolytic mechanism (Spencer *et al.*, 1996, 2007b; Spencer and Bazer, 2004; Bazer *et al.*, 2010). The antiluteolytic effects of IFNT are to inhibit transcription of the *estrogen receptor alpha (ESR1)* gene in sheep and *oxytocin receptor (OXTR)* gene in both sheep and cattle specifically in the endometrial LE. The absence of OXTR in the endometrium prevents the release of luteolytic pulses of PGF2 $\alpha$ , thereby sustaining lifespan of the CL and P4 production. Although IFNT inhibits OXTR expression, it does not inhibit expression of *PTGS2*, which is important for the generation of PGs that are critical regulators of conceptus elongation during early pregnancy (Dorniak *et al.*, 2011). In addition to antiluteolytic effects, IFNT acts in a paracrine manner on the endometrium to induce or enhance expression of ISGs that are hypothesized to regulate uterine receptivity and conceptus elongation and implantation (Hansen *et al.*, 1999, 2010; Spencer *et al.*, 2008; Bazer *et al.*, 2009a).

#### Classical type I IFN-stimulated genes in the endometrium

A number of transcriptional profiling experiments conducted with human cells, ovine endometrium, bovine endometrium, and bovine peripheral blood lymphocytes have elucidated classical ISG induced by IFNT during pregnancy (Spencer *et al.*, 2007a, 2008; Ott and Gifford, 2010; Forde *et al.*, 2011b; Bauersachs *et al.*, 2012). In cattle, comparisons of days 15 to 18 pregnant and non-pregnant or cyclic endometria revealed conceptus effects on endometrial gene expression, particularly the induction or up-regulation of classical IFN-stimulated genes (ISGs; Bauersachs *et al.*, 2006, 2012; Forde *et al.*, 2009, 2011b; Cerri *et al.*, 2012; Forde and Lonergan, 2012). In sheep, *ISG15* (ISG15 ubiquitin-like modifier) is expressed in LE of the ovine uterus on days 10 or 11 of the estrous cycle and pregnancy, but are undetectable in LE by days 12 to 13 of pregnancy (Johnson *et al.*, 1999b). In response to IFNT from the elongating conceptus, *ISG15* is induced in the stratum compactum stroma and GE by days 13 to 14, and expression extends to the stratum spongiosum stroma, deep glands, and myometrium as well as resident immune cells of the ovine uterus by days 15 to

16 of pregnancy (Johnson *et al.*, 1999b, 2000). As IFNT production by the conceptus trophectoderm declines, expression of ISG in the stroma and GE also declines, but some remain abundant in endometrial stroma and GE on days 18 to 20 of pregnancy. Similar temporal and spatial alterations in *ISG15* expression occur in the bovine uterus during early pregnancy (Johnson *et al.*, 1999a; Austin *et al.*, 2004).

*In vivo* studies revealed that the majority of classical ISG (*B2M*, *GBP2*, *IFI27*, *IFIT1*, *ISG15*, *IRF9*, *MIC*, *OAS*, *RSAD2*, *STAT1*, and *STAT2*) are not induced or up-regulated by IFNT in endometrial LE or sGE of the ovine uterus during early pregnancy (Johnson *et al.*, 1999b, 2001; Choi *et al.*, 2001, 2003; Song *et al.*, 2007). This finding was initially surprising, because all endometrial cell types express *IFNAR1* (interferon [alpha, beta, and omega] receptor 1) and *IFNAR2* subunits of the common Type I IFN receptor (Rosenfeld *et al.*, 2002). Further, bovine endometrial, ovine endometrial, and human 2fTGH fibroblast cells were used to determine that IFNT activates the canonical janus kinase-signal transducer and activator of transcription-interferon regulatory factor (JAK-STAT-IRF) signaling pathway used by other Type I IFNs (Stark *et al.*, 1998). About the same time, it was discovered that IRF2, a potent transcriptional repressor of ISG (Taniguchi *et al.*, 2001), is expressed specifically in the endometrial LE and sGE and represses transcriptional activity of IFN-stimulated response element (ISRE)-containing promoters (Spencer *et al.*, 1998; Choi *et al.*, 2001). In fact, all components of the ISGF3 transcription factor complex (*STAT1*, *STAT2*, *IRF9*) and other classical ISGs (*B2M*, *GBP2*, *IFI27*, *IFIT1*, *ISG15*, *MIC*, *OAS*) contain one or more ISRE in their promoters. Thus, IRF2 in LE appears to restrict IFNT induction of most classical ISG to stroma and GE of the ovine uterus (Dorniak *et al.*, 2013a). The silencing of *MIC* and *B2M* genes in endometrial LE or sGE during pregnancy may be a critical mechanism preventing immune rejection of the semi-allogeneic conceptus (Choi *et al.*, 2003). As IRF2 is not expressed in other uterine cell types, classical ISG are substantially increased in the endometrial stroma, GE and immune cells by IFNT from the conceptus during early pregnancy by IFNT. Of particular note, several reports indicate induction or increases in ISGs in peripheral blood lymphocytes and the CL during pregnancy of sheep and cattle or in ewes receiving intrauterine injections of IFNT (Hansen *et al.*, 2010; Ott and Gifford, 2010). Recent evidence indicates that IFNT traffics out of the uterus to exert systemic effects that alter maternal physiology, such as function of the CL (Bott *et al.*, 2010; Hansen *et al.*, 2010).

One challenge has been to determine which of the large number of classical ISGs induced in the endometrium by IFNT have a biological role in conceptus-endometrial interactions, given that they have traditionally been associated with cellular antiviral



responses as the main function of Type I IFN is to inhibit viral infection (Pestka, 2007). One classical ISG with reported biological effects on trophectoderm growth and adhesion in ruminants is *CXCL10* [chemokine (C-X-C motif) ligand 10; alias IP-10], a member of the C-X-C chemokine family that regulates multiple aspects of inflammatory and immune responses primarily through chemotactic activity toward subsets of leukocytes (Nagaoka *et al.*, 2003a, b). ISG15 conjugates to intracellular proteins through a ubiquitin-like mechanism (Hansen *et al.*, 1999), and deletion of *Isg15* in mice results in 50% pregnancy loss manifest during early placentation (Ashley *et al.*, 2010). In addition, MX proteins are thought to regulate secretion through an unconventional secretory pathway (Toyokawa *et al.*, 2007). The enzymes which comprise the 2',5'-oligoadenylate synthetase (OAS) family regulate ribonuclease L antiviral responses and may play additional roles in control of cellular growth and differentiation (Johnson *et al.*, 2001).

#### *Non-classical IFNT-stimulated genes in the endometrium*

Although IFNT is the only known IFN to act as the pregnancy recognition signal, IFN appear to have a biological role in uterine receptivity, decidualization, and placental growth and development in primates, ruminants, pigs, and rodents (Hansen *et al.*, 1999; Bazer *et al.*, 2009a). Transcriptional profiling of human U3A (STAT1 null) cells and ovine endometrium, as well as candidate gene analyses were used to discover novel 'non-classical' ISG in the endometrial LE during pregnancy such as *WNT7A* (wingless-type MMTV integration site family, member 7A), *LGALS15*, *CTSL*, *CST3*, *HSD11B1*, and *IGFBP1* (Kim *et al.*, 2003a; Song *et al.*, 2005, 2006; Gray *et al.*, 2006; Satterfield *et al.*, 2006).

Subsequently, a series of transcriptomic and candidate gene studies found that IFNT stimulates expression of a number of elongation- and implantation-related genes that are initially induced by P4 (*CST3*, *CST6*, *CTSL*, *GRP*, *HSD11B1*, *IGFBP1*, *LGALS15*, *SLC2A1*, *SLC2A5*, *SLC5A11*, *SLC7A2*) specifically in the endometrial LE, sGE, and/or GE (Spencer *et al.*, 2007a, 2008; Bazer *et al.*, 2009a, b; Fig. 1). None of those genes are classical Type I ISG, and they are referred to as 'non-classical or novel' ISG. Indeed, IFNT stimulation of those non-classical ISG requires induction by P4 and loss of PR in the epithelia. Importantly, all of the non-classical ISG encode factors that have actions on the trophectoderm (proliferation, migration, attachment and/or adhesion, nutrient transport) important for conceptus elongation (Table 1). The effects of IFNT in the bovine endometrium are not as well understood in terms of non-classical ISGs, but recent studies have started to unravel those effects in cattle (Forde *et al.*, 2011b, 2012a; Bauersachs *et al.*, 2012).

Given that the critical signaling components of the JAK-STAT signaling system (STAT1, STAT2, IRF9) are not expressed in endometrial LE or sGE (Choi *et al.*, 2001), IFNT must utilize a noncanonical, STAT1-independent signaling pathway to regulate expression of genes in endometrial LE and sGE of the ovine uterus. The noncanonical pathway mediating IFNT stimulation of genes in the endometrial LE and sGE has not been entirely elucidated, but other Type I IFN utilize mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) cascades (Platanias, 2005). Recent evidence indicates that IFNT activates distinct epithelial and stromal cell-specific JAK, epidermal growth factor receptor, MAPK (ERK1/2), PI3K-AKT, and/or Jun N-terminal kinase (JNK) signaling modules to regulate expression of PGE<sub>2</sub> receptors in the endometrium of the ovine uterus or in ovine uterine LE cells *in vitro* (Banu *et al.*, 2010; Lee *et al.*, 2012). As discussed subsequently, recent evidence indicates that PTGS2-derived PGs and HSD11B1-derived cortisol are part of the noncanonical pathway of IFNT action on the endometrium in sheep (Dorniak *et al.*, 2011, 2012a, b, 2013b).

#### **Prostaglandin regulation of endometrial function and conceptus elongation**

Results of recent studies in sheep clearly support the concept that PGs regulate expression of elongation- and implantation-related genes in the endometrial epithelia of ruminants during early pregnancy and are involved in conceptus elongation (Simmons *et al.*, 2009, 2010; Dorniak *et al.*, 2011; Fig. 1 and 2). The conceptus and endometria synthesize a variety of PGs during early pregnancy in both sheep and cattle (Lewis *et al.*, 1982; Lewis and Waterman, 1983; Lewis and Waterman, 1985; Lewis, 1989; Charpigny *et al.*, 1997a, b). The endometrium and uterine lumen also contains and produces substantially more PG during early pregnancy than during the estrous cycle (Ellinwood *et al.*, 1979; Marcus, 1981; Ulbrich *et al.*, 2009). Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) or PTGS2 is the dominant cyclooxygenase expressed in both the endometrium and trophectoderm of the elongating conceptus (Charpigny *et al.*, 1997a, b). Although the antiluteolytic effects of IFNT are clearly to inhibit expression of the *OXTR* in the endometrial LE and sGE of early pregnant ewes, it does not impede up-regulation of PTGS2, a rate-limiting enzyme in PG synthesis (Charpigny *et al.*, 1997b; Kim *et al.*, 2003b). As illustrated in Fig. 1, *PTGS2* expression appears between days 10 and 12 post-estrus and mating in the endometrial LE and sGE and is induced by ovarian P4 (Charpigny *et al.*, 1997b; Simmons *et al.*, 2010). In the bovine uterus, PTGS2 is also not down-regulated in endometria of early pregnant cattle, but rather is up-regulated by IFNT (Arosh *et al.*, 2004; Emond *et al.*, 2004); indeed, IFNT acts as a molecular switch that



stimulates PGE<sub>2</sub> production in the bovine endometrium (Krishnaswamy *et al.*, 2009). Further, Type I IFNs were found to stimulate phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and synthesis of PGE<sub>2</sub> and PGF<sub>2</sub>α in several different cell types over 25 years ago (Fitzpatrick and Stringfellow, 1980; Fuse *et al.*, 1982).

Prostaglandins clearly regulate endometrial functions and conceptus elongation during early pregnancy (Simmons *et al.*, 2010; Dorniak *et al.*, 2011, 2012a, b; Table 1 and Fig. 2). In sheep, PTGS2 activity in

the endometrium is stimulated by IFNT, and PTGS2-derived PG were found to mediate, in part, the effects of P4 and IFNT on the endometrium of the ovine uterus. In those studies, the abundance of *HSD11B1* and *IGFBP1* mRNA in the endometrium was considerably reduced by intrauterine infusion of meloxicam, a selective PTGS2 inhibitor. Both *HSD11B1* and *IGFBP1* are upregulated by PGs in the ovine placenta and human uterine decidua, respectively (Strakova *et al.*, 2000; Michael *et al.*, 2003; Michael and Papageorgiou, 2008).

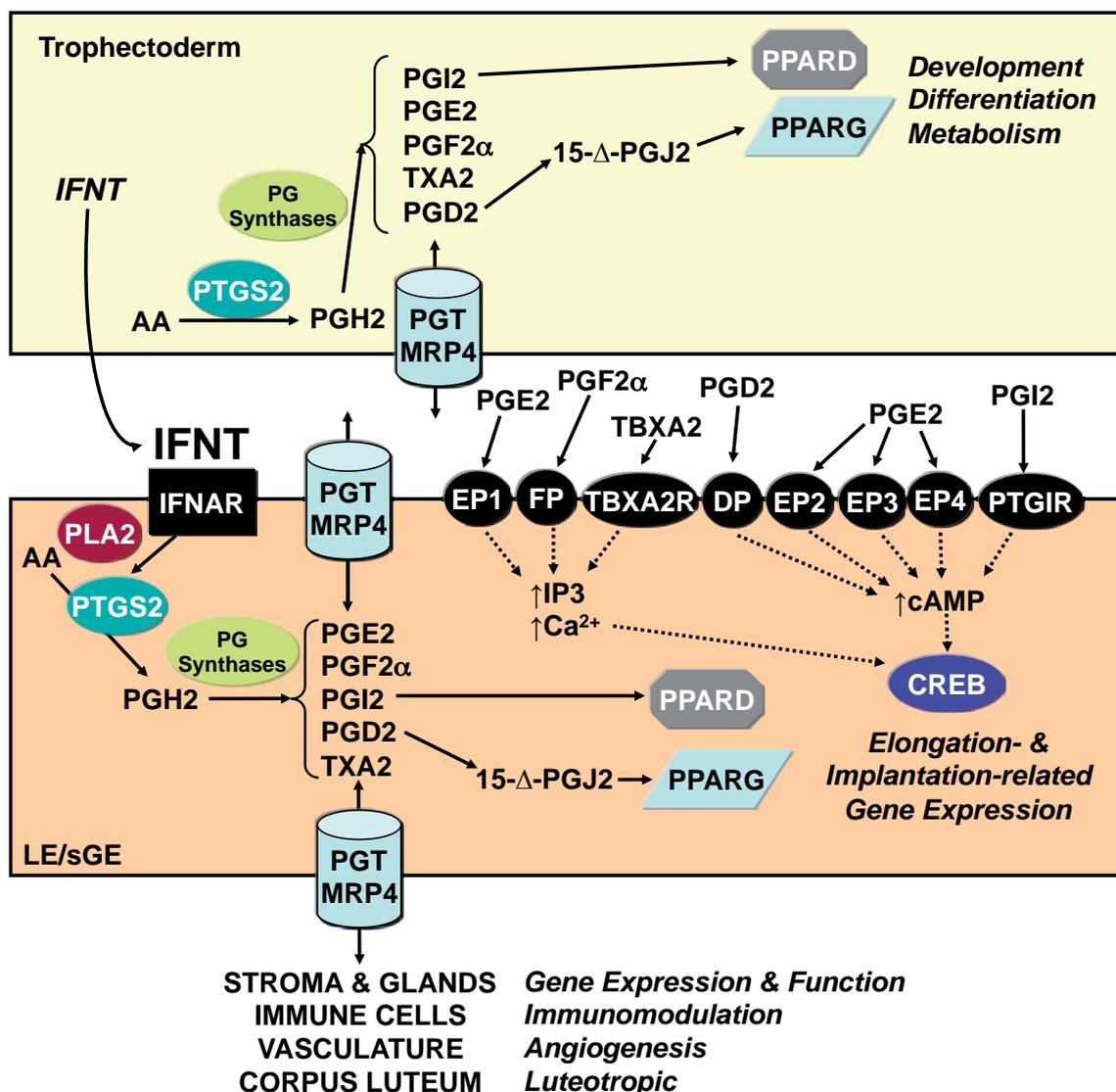


Figure 2. Schematic illustrating working hypothesis of the biological role of interferon tau (IFNT) and prostaglandins (PGs) in uterine function and conceptus elongation during early pregnancy in sheep. See text for detailed description. Legend: ABCC4, ATP-binding cassette, sub-family C (CFTR/MRP), member 4; CREB, cAMP responsive element binding protein; IFNAR, interferon (alpha, beta, and omega) receptor; DP, prostaglandin D receptor (PTGDR); EP, prostaglandin E receptor (PTGER); FP, prostaglandin F receptor (PTGFR); IP, prostaglandin I receptor (PTGIR); PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PPARD, peroxisome proliferator-activated receptor delta; PPARG, peroxisome proliferator-activated receptor gamma; PTGS2, prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase); PG Synthases, prostaglandin synthases (AKR1C3, PTGDS, PTGES, PTGFS, PTGIS, TBXAS); SLCO2A1, solute carrier organic anion transporter family, member 2A1 (prostaglandin transporter); TBXA2R, thromboxane A<sub>2</sub> receptor.



Prostaglandins are essential for conceptus elongation, as intrauterine infusions of meloxicam prevented conceptus elongation in early pregnant sheep (Simmons *et al.*, 2010; Dorniak *et al.*, 2011). The elongating conceptuses of both sheep and cattle synthesize and secrete more PG than the underlying endometrium (Lewis *et al.*, 1982; Lewis and Waterman, 1983; Lewis, 1989). Thus, PG levels are much greater in the uterine lumen of pregnant as compared with cyclic or nonpregnant cattle (Ulbrich *et al.*, 2009). Day 14 sheep conceptuses *in vitro* release mainly cyclooxygenase metabolites including PGF2 $\alpha$ , 6-keto-PGF1 $\alpha$  (i.e., a stable metabolite of PGI<sub>2</sub>), and PGE<sub>2</sub> (Charpigny *et al.*, 1997a), and day 16 conceptuses produce substantially more of those PG than day 14 conceptuses (Lewis and Waterman, 1985). Given that membrane and nuclear receptors for PGs are present in all cell types of the endometrium and conceptus during early pregnancy (Cammis *et al.*, 2006; Dorniak *et al.*, 2011), PTGS2-derived PGs from the conceptus likely have paracrine, autocrine, and perhaps intracrine effects on endometrial function and conceptus development during early pregnancy. Indeed, expression of *PTGS2* in biopsies of day 7 bovine blastocysts is a predictor of the successful development of that blastocyst to term and delivery of a live calf (El-Sayed *et al.*, 2006). Recently, Dorniak and coworkers (Dorniak *et al.*, 2012a) infused PGE<sub>2</sub>, PGF2 $\alpha$ , PGI<sub>2</sub>, or IFNT at the levels produced by the day 14 conceptus into the uterus of cyclic ewes. In that study, expression of *GRP*, *IGFBP1*, and *LGALS15* were increased by PGE<sub>2</sub>, PGI<sub>2</sub>, and IFNT, but only IFNT increased *CST6* (Table 1). Differential effects of PG were also observed for *CTSL* and its inhibitor *CST3*. For glucose transporters, IFNT and all PG increased *SLC2A1*, but only PG increased *SLC2A5* expression, whereas *SLC2A12* and *SLC5A1* were increased by IFNT, PGE<sub>2</sub>, and PGF2 $\alpha$ . Infusions of all PG and IFNT increased the amino acid transporter *SLC1A5*, but only IFNT increased *SLC7A2*. In the uterine lumen, only IFNT increased glucose levels, and only PGE<sub>2</sub> and PGF2 $\alpha$  increased total amino acids (Dorniak *et al.*, 2012a). Thus, available results support the idea that PG and IFNT from the conceptus coordinately regulate endometrial functions important for growth and development of the conceptus during the peri-implantation period of pregnancy (Dorniak *et al.*, 2013a). In fact, pregnancy rates were substantially reduced in heifers that received meloxicam, a partially selective inhibitor of PTGS2, on day 15 after insemination (Erdem and Guzeloglu, 2010). Thus, PGs are critical regulators of conceptus elongation and implantation in ruminants, as they are for blastocyst implantation and decidualization during pregnancy in mice, rats, hamsters, mink, and likely humans (Dey *et al.*, 2004; Wang and Dey, 2006; Kennedy *et al.*, 2007).

## Conclusions

The individual, additive and synergistic actions of P4, IFNT, and PGs regulate expression of elongation- and implantation-related genes in the endometrial epithelia and that P4 and PGs are essential regulators of conceptus elongation in ruminants. The outcome of these carefully orchestrated changes in gene expression is secretion or transport of substances (e.g., glucose, amino acids, proteins) from the endometrium into the uterine lumen that govern conceptus survival and elongation via effects on trophoblast proliferation, migration, attachment, and adhesion. Recent studies indicate that some, but not all, of the same mechanisms, pathways and factors regulate conceptus elongation in cattle are conserved between cattle and sheep (Bauersachs *et al.*, 2008; Spencer *et al.*, 2008; Forde *et al.*, 2011b; Forde and Lonergan, 2012). One important area of future research is determining which endometrial genes and products are critical determinants of uterine receptivity and early pregnancy success. This knowledge should be useful to develop genetic tools essential to select animals for enhanced fertility. Improvement of functional traits using conventional approaches of quantitative genetics is difficult, because most reproductive traits are complex (polygenic) with low heritability (Weigel, 2006; Veerkamp and Beerda, 2007). McMillan and Donnison (1999) summarized a novel approach for experimentally identifying high and low fertility heifers based on early pregnancy success using serial transfer of *in vitro*-produced embryos. Of note, those investigators suggested that a failure in the mechanism involved in conceptus elongation and maternal recognition of pregnancy was a major cause of early pregnancy loss in low fertility heifers (McMillan and Donnison, 1999; Peterson and Lee, 2003). Accordingly, the selected high fertility heifers would have a uterus that was superior in the ability to support growth and development of the conceptus. Thus, natural variation in early pregnancy rates in cattle can be used to define genes and pathways important for endometrial receptivity and essential for early pregnancy loss and success. Other ruminant models to understand endometrial receptivity and pregnancy loss include: (a) the UGKO ewe (Gray *et al.*, 2002); (b) heifers versus cows (Berg *et al.*, 2010); (c) non-lactating versus lactating cows (Cerri *et al.*, 2012); (d) advanced versus delayed post-ovulatory rise in P4 (Lonergan, 2011; Forde and Lonergan, 2012); and (e) recessive lethal mutations that manifest in defective conceptus elongation and/or epiblast formation (Charlier *et al.*, 2012). A systems biology approach is necessary to understand the multifactorial phenomenon of recurrent pregnancy loss and provide a basis for new strategies to improve pregnancy outcomes, fertility, and reproductive efficiency in ruminant livestock.



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## Managing gestation in cattle

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### Abstract

Once a cow becomes pregnant, the effect of pregnancy loss on its reproductive cycle is a topic of great interest for dairy herds. This paper reviews the factors of a non-infectious nature that affect the pregnancy maintenance during the late embryonic/early fetal period. Some clinical suggestions on bovine neosporosis and coxiellosis are also highlighted.

**Keywords:** coxiellosis, early fetal period, late embryonic period, neosporosis, pregnancy loss.

### Introduction

#### Factors of non-infectious nature related to late embryonic/early fetal loss

Dairy herds are under ever-increasing pressure to improve their efficiency and primary attention is usually directed towards postpartum reproductive disorders. However, it is also of great interest to understand the effects of pregnancy loss on the reproductive cycle once a cow is pregnant. Following a positive pregnancy diagnosis, late embryonic/early fetal loss is becoming the most common complication of gestation in high producing dairy herds. In the cow, the embryonic period of gestation extends from conception until the end of the differentiation stage (about 45 days), and the fetal period spans from day 45 of gestation to parturition (Committee on Bovine Reproductive Nomenclature, 1972). Placentation finishes before day 60 of gestation, the period in which pregnancy is considered to be firmly established and the chances of loss are reduced (Ball, 1997). In dairy cattle, the risk of early fetal loss seems to increase under conditions of intensive management (Santos *et al.*, 2004; López-Gatius *et al.*, 2009). An early fetal loss of 10-12% is a commonly accepted figure. Losses, however, are further aggravated in specific populations such as: lactating parous cows, which show a 3.6-times more of pregnancy loss than pregnant heifers (Labèrnia *et al.*, 1996; López-Gatius *et al.*, 2004b); cows with previous postpartum disorders such as placenta retention or pyometra, with a pregnancy loss of 1.8 and 2.6 times higher respectively, than cows not suffering the disorder (López-Gatius *et al.*, 1996); cows inseminated by a particular bull, which show 2 to 22 times higher risk of pregnancy loss

(López-Gatius *et al.*, 2002, 2004c, 2007); cows bearing twins, with a pregnancy loss of 3 to 7 times higher for twin pregnancies than single pregnancies (López-Gatius *et al.*, 2002, 2006; García-Ispuerto *et al.*, 2006; Silvaldel-Rio *et al.*, 2009). Moreover, in warm countries such as Spain, summer heat stress is a major factor associated dramatically not only with conception (Labèrnia *et al.*, 1998; López-Gatius, 2003; García-Ispuerto *et al.*, 2007) but also with fetal loss (López-Gatius *et al.*, 2004c, d; García-Ispuerto *et al.*, 2006). Suboptimal concentrations of progesterone in blood related to high milk production (Bech-Sabat *et al.*, 2008; Rhinehart *et al.*, 2009) could also explain some of these losses during the late embryonic/early fetal period (Ayad *et al.*, 2007; Gábor *et al.*, 2008) and why rates can exceed 20% in high production systems (Cartmill *et al.*, 2001; Grimard *et al.*, 2006). The fact that intravaginal progesterone supplementation may reduce the incidence of pregnancy loss during the early fetal period supports this idea (López-Gatius *et al.*, 2004d; Alnimer and Lubbadah, 2008). In fact, early fetal loss, peaking between days 40 and 50 of gestation (López-Gatius *et al.*, 2004c; Santos *et al.*, 2004), is becoming the most common complication of gestation in high producing dairy cows in our geographical area, where more than 90% of pregnancy losses following pregnancy diagnosis occur usually before day 90 (López-Gatius and Garcia-Ispuerto, 2010; López-Gatius, 2012).

#### Factors of infectious nature related to pregnancy loss

*Neosporacanium* is a protozoan parasite with a wide host range but with a preference for cattle and dogs. Since the description of *N. caninum* as a new genus and species in 1988, bovine neosporosis has become a disease of international concern as it is among the main causes of abortion in cattle (Dubey and Lindsay, 1996; Dubey and Schares, 2011). At present there is no effective treatment or vaccine. *Neosporacanium* is one of the most efficiently transplacentally transmitted organisms in cattle. Up to 95% of calves are born infected (Dubey *et al.*, 2007). The majority of calves born from infected mothers are clinically normal, but they are infected for life and *N. caninum* infection can be maintained over several generations by vertical transmission (Pabón *et al.*, 2007). Abortion is the main clinical manifestation of bovine neosporosis with most abortions occurring at 5-7 months

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of gestation (Dubey *et al.*, 2007). For example, in our geographical area of study, based on the odds ratio, the risk of abortion was found to be 12-19 times higher in *Neospora*-seropositive dairy cows than in seronegative cows, ranging from 30 to 44% in seropositive animals (López-Gatius *et al.*, 2004a, b), and maintaining a similar risk of abortion over several years (Pabón *et al.*, 2007). In fact, in commercial dairy herds routinely examined by us in a reproductive management program, 0% is a common figure for the abortion rate during the second and third terms of gestation for *Neospora*-seronegative animals (Almería and López-Gatius, 2013). However, chronic *N. caninum* infection prior to pregnancy seems to be not responsible for abortion before day 90 of gestation (López-Gatius *et al.*, 2004b).

*Coxiellaburnetii* is an intracellular bacterium spread worldwide that causes Q fever in animals and also in humans. Domestic ruminants such as cattle, goats and sheep are considered to be the primary reservoir species for exposure of humans (Arricau-Bouvery and Rodolakis, 2005). Bacteria have been found in placenta or aborted fetuses (Parisi *et al.*, 2006), and the main clinical manifestations are late abortion (Woldehiwet, 2004), infertility (To *et al.*, 1998) and metritis and placenta retention (López-Gatius *et al.*, 2012). Interactions throughout gestation between *Neospora*- and *Coxiella*-infection have been furthermore described in cattle. Both the *N. caninum* and *C. burnetii* infection or the presence of both modifies endocrine patterns throughout gestation. Cows seropositive to both *Neospora* and *Coxiella* had higher plasma progesterone levels (García-Ispuerto *et al.*, 2010), and stable *Coxiella*-seropositivity (García-Ispuerto *et al.*, 2011).

This presentation, based mainly on our results in northeast Spain, expresses our views on factors affecting early fetal loss in high producing dairy herds and therapeutic approaches to the disorder. Some clinical suggestions on bovine neosporosis and coxiellosis are also highlighted.

### Parity status and pregnancy losses

Grouping data derived from two studies reported that (Labèrnia *et al.*, 1996; López-Gatius *et al.*, 2004b) 2.6% of pregnant heifers suffered fetal loss, whereas 9% losses were registered for 5124 lactating pregnant cows. Our values are comparable to those obtained between 1949 and 1955: 2.5% of losses for heifers and 13% for multiparous Holstein-Friesian cows (Mares *et al.*, 1961), and to those compiled from the literature by Santos *et al.* (2004): pregnancy loss was 10.7% for lactating cows and 4.2% for dairy heifers. These findings suggest that genetic selection for high milk production did not affect the incidence of losses during the last decades. Parturition and metabolic stress associated with lactation seems to compromise fetal survival.

### The problem of twin pregnancies

Using cows carrying singletons as reference and compiling five studies (López-Gatius *et al.*, 2002, 2004c, 2006; López-Gatius and Hunter, 2005; García-Ispuerto *et al.*, 2006), the minimum odds ratio registered for pregnancy losses for twin pregnancies was 3.1 compared with cows bearing singletons. In the warm period, up to 54% of losses were registered in twin pregnancies (López-Gatius *et al.*, 2004c).

Since genetics appears to be a major regulatory factor for twinning rates last decades (Johanson *et al.*, 2001), it is reasonable to suggest that increased twinning is a consequence of selection for milk yield, but aside from genetic progress, improvements in nutrition and management practices have led also to a continuous increase in the milk yield. Probably, the improved management at the farm level has diminished the risk of embryo loss in twin pregnancies and thus raised the twinning rate. It is therefore foreseeable that over the years to come, the twinning rate will continue to increase along with milk production.

### Twin reduction

Twin pregnancies are undesirable in dairy cattle since they increase not only the risk of early pregnancy loss, but also have many negative effects such as increased abortion, dystocia, retained placenta, calf mortality, occurrence of freemartins, postpartum therapy, and longer rebreeding intervals (Nielen *et al.*, 1989; Andreu-Vázquez *et al.*, 2012a). Such negative effects of twinning might be diminished by reducing the embryo number in dairy cows.

We evaluated embryo reduction methods by manual rupture of the amniotic vesicle of a twin embryo (López-Gatius, 2005; Andreu-Vázquez *et al.*, 2011) or bytrans vaginal ultrasound-guided embryo aspiration (Andreu-Vázquez *et al.*, 2012b). Although these techniques should be further investigated, both can provide in the future a satisfactory way for twin reduction in dairy cattle.

It should be noted here the fact that lactation number (López-Gatius *et al.*, 2005a), previous twinning, as well as environmental factors, such as photoperiod and season and management related to synchronization protocols affect significantly the incidence of twin pregnancies (Andreu-Vázquez *et al.*, 2012c).

Spontaneous twin reduction has also been described in cows that remain pregnant. Interestingly, most embryonic mortality (one of the two embryos) occurs at around days 35-40 of gestation so that the fate of twin pregnancies progressing normally until day 60 is either the delivery of both twins or abortion. No fetal death appears to occur after this time point (López-Gatius and Hunter, 2005; López-Gatius *et al.*, 2010). Thus, the detection of live twins on day 60 of gestation has enormous implications for the management policy



of a herd. For example, since twins are delivered up to seven days earlier than singletons, the dry-off period can be advanced several days for non-aborting cows carrying twins. Additional care at parturition can further reduce the risk of calf mortality in a twin pregnancy.

#### **Additional corpus luteum, a factor associated with reduced fetal loss**

Additional corpus luteum has demonstrated to be a very strong factor reducing fetal loss in pregnancies with a greater number of corpora lutea than the number of embryos. On a total of 363 pregnant cows with an additional corpus luteum derived from five studies (López-Gatius *et al.*, 2002, 2004c, 2006; García-Ispierto *et al.*, 2006; Bech-Sabat *et al.*, 2008), 1.7% suffered fetal loss, whereas 9.9% losses were registered for 3643 pregnant animals with no additional corpus luteum. However, spontaneous reduction of the additional corpus luteum in multiple ovulating cows that remain pregnant can occur under certain forms of stress (López-Gatius *et al.*, 2010).

#### **Therapeutic approaches to early fetal loss**

Progesterone is required for supporting gestation; it influences secretor functions of trophoblast and pituitary during the first trimester of gestation (Ayad *et al.*, 2007). However, one of the consequences of high milk production is an increased metabolic rate linked to a greater dry matter intake. This process reduces plasma concentrations of steroid hormones such as progesterone (Sangsrivong *et al.*, 2002). In fact, milk production can affect negatively plasma progesterone concentrations at the onset of the fetal period (Bech-Sabat *et al.*, 2008; Rhinehart *et al.*, 2009). Therefore, it seems reasonable to suppose that one of the causes of early fetal loss in high producing dairy cows could be the suboptimal concentrations of progesterone. Thus, strategies that induce the formation of an additional corpus luteum may help to increase progesterone levels in high producers. However, although treatment with GnRH at AI (López-Gatius *et al.*, 2006) and with GnRH or hCG at pregnancy diagnosis (Bartolomé *et al.*, 2006; Stevenson *et al.*, 2008) clearly increased the number of additional corpora lutea, treatment did not reduce fetal loss in any of the studies.

In order to test the hypothesis that suboptimal progesterone concentrations may compromise conceptus development, we treated pregnant cows with progesterone at pregnancy diagnosis during four weeks (López-Gatius *et al.*, 2004d). The risk of pregnancy loss was 2.4 times higher in non-treated cows ( $n = 549$ ) than in treated ones ( $n = 549$ ). Under these conditions, intravaginal progesterone supplementation has the potential to reduce the incidence of pregnancy loss during the early fetal period. We could speculate that progesterone supplementation at pregnancy diagnosis

could favor placentation, fetal development, or both.

In a more recent study in two herds with high incidence of fetal loss (Bech-Sabat *et al.*, 2009), in cows with one single corpus luteum, the probability of pregnancy loss decreased by a factor of 0.51 in cows treated with progesterone, compared to the GnRH treatment. However, in cows with two or more corpora lutea, progesterone treatment increased the likelihood of pregnancy loss by a factor of 3, compared to GnRH treatment. These results suggest that at pregnancy diagnosis (i.e.: days 28-34), it is so important to register the number of corpora lutea as the number of embryos.

The practical implications of these findings are that in herds with a high incidence of early fetal loss of a non-infectious nature, treatment at the time of pregnancy diagnosis with progesterone in cows with one single corpus luteum and with GnRH in cows carrying twins should offer considerable benefits (Bech-Sabat *et al.*, 2010).

#### **Crossbred pregnancies reduce the abortion risk in *Neosporacanium*-infected dairy cows**

An important finding of our studies addressing the control and prevention of cattle neosporosis in dairy cattle is that the use of beef bull semen reduces the risk of *N. caninum*-associated abortion (López-Gatius *et al.*, 2005b). Our retrospective analysis of the effects of different cross-breed pregnancies on the abortion risk in *Neospora*-infected dairy cows returned abortion rates from 32% of 482 cows inseminated with Holstein-Friesian semen, to 22% of 49 cows inseminated with Charolais semen, 20% of 191 cows inseminated with Belgium Blue semen, 19% of 89 cows inseminated with Piedmontese semen, and 10% of 304 cows inseminated with Limousin semen (Almería *et al.*, 2009).

#### **Questions regarding bovine coxiellosis**

Reproductive disorders related to coxiellosis are frequently described in cattle, but the results are often inconsistent. For example, based on serology, *Coxiella*-seropositivity was linked to placenta retention, to changes in the interval from parturition to conception (with the lowest interval parturition-conception for cows with low level of seropositivity), early pregnancy (cows becoming pregnant before day 90 postpartum), and maintenance of gestation during the early fetal period, while it failed to affect rates of abortion after day 90 of gestation or stillbirth (López-Gatius *et al.*, 2012). Extensive studies are needed to understand better the effect of *Coxiella*-infection in the dairy herds.

#### **Concluding remarks**

Once a cow has been diagnosed pregnant, early fetal loss is becoming the most common complication of pregnancy in high producing dairy herds. Factors



strongly affecting early fetal loss are parity (cows versus heifers), semen-providing bull, warm season, and twin pregnancies, whereas the presence of an additional corpus luteum has been identified as a strong positive factor favoring pregnancy maintenance. Progesterone and GnRH treatment have the potential to reduce the incidence of pregnancy loss in cows with one or two or more corpora lutea, respectively, in herds with a high incidence of early fetal loss of a non-infectious nature. From a practical point of view, assessment of normal development of gestation on day 60 after insemination is suggested.

Different crossbreed pregnancies carry different abortion risks in *Neospora*-infected dairy cows. The use of beef bull semen (especially Limousin) dramatically reduces the risk of abortion.

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## Dietary lipid supplementation on cow reproductive performance and oocyte and embryo viability: a real benefit?

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### Abstract

The practice of “fat feeding” has become common in the dairy industry in a number of countries. There are several ideas as to how dietary lipids could influence reproductive performance. Highly saturated triacylglycerols (TAG), like palm oil, can increase milk yield but may aggravate negative energy balance and consequently impair fertility when fed during the first weeks postpartum. However, priming the lipid oxidation in the liver by feeding saturated lipid sources during the dry period has recently been shown to be a potentially promising strategy to mitigate fat mobilization and liver accumulation postpartum. Furthermore, polyunsaturated free fatty acids (FFA), such as omega-3 fatty acids and conjugated linoleic acids are fed to reduce the ‘de novo’ fatty acid synthesis in the udder and thus the milk TAG content, which may be of modest benefit for overall energy balance. Furthermore, omega-6 and -3 polyunsaturated FFA are reported to alter follicular growth, steroid synthesis and prostaglandin metabolism in the ovary and endometrium, respectively. Omega-6 FFA are believed to have proinflammatory and thus PGF2 $\alpha$ -stimulating properties rendering them extra value as “neutraceutical” early postpartum, while omega-3 FFA can weaken this inflammatory potency, leading to a higher chance of survival of the embryo when supplemented during the periconceptual period. Unfortunately, research results rarely provide a consensus in this perspective. The consequences of these fat feeding strategies on oocyte and embryo quality remain an intriguing issue for debate. Dietary lipid supplementation may alter the microenvironment of the growing and maturing oocyte, of the early and older embryo and thus may affect reproductive outcome. We recently reported that dietary induced hyperlipidemic conditions can be harmful for embryo development and metabolism. However, to date, research results remain somewhat conflicting most probably due to differences in fat sources used in diet, and duration of supplementation and in experimental set up.

**Keywords:** dietary fat supplementation, energy balance, oocyte and embryo quality, reproduction.

### Introduction

The dairy cow industry has changed dramatically over the past decades. Per-cow milk yields have increased dramatically as a combined result of improvements in animal management, nutrition, and genetics. A prerequisite for good lactation performance during a cow’s life span is the production of offspring at regular intervals. Consequently, reproductive efficiency is fundamental for the modern dairy industry, as fertility influences average daily milk production, average days in milk, number of calves born per year, the generational interval, and ultimately the farmer’s income (Leroy and de Kruif, 2006; Inchaisri *et al.*, 2011). Many studies have reported a worrisome decrease in the reproductive performance of dairy cows in recent decades, and this problem appears to affect all countries benefiting from high yielding dairy herds (for review see: Leroy and de Kruif, 2006). Reproductive failure is a major reason for rapid culling, threatening longevity of dairy cows and the sustainability of modern dairying. Furthermore, only an optimal reproduction at herd level guarantees an acceptable environmental ecological foot print of milk production (Garnsworthy, 2004). Reproductive failure in dairy cows is a multifactorial and complex problem. Calving under hygienic conditions and devoid of stress should guarantee optimal uterine involution and the absence of endometritis. Good feeding strategies (composition, quantity, palatability, availability, and the access of the feed) are also important. More and more farmers know that keeping the cows eating throughout this sensitive transition period represents their greatest challenge (Janovick and Drackley, 2010). Any drop in appetite and thus in dry matter intake increases the pressure on the cow’s metabolic health. Recently, Walsh *et al.* (2011) elegantly considered all of the key steps in dairy cow reproduction and listed the pathways on how reproductive failure can originate, as well as provided known risk factors. The interactions between early postpartum negative energy balance (NEB), and the hypothalamus-pituitary-ovary-uterus axis have been particularly well studied (Ducker *et al.*, 1985; Lucy, 2001; Armstrong *et al.*, 2002; Butler, 2003). Disrupted endocrine signalling delays resumption of ovarian

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cyclicity postpartum; a relationship well-recognized as a major factor in dairy cow reproductive failure (Opsomer *et al.*, 1998; Roche, 2006; Vanholder *et al.*, 2006). However, attention has recently shifted to the widely reported fall in conception rates (Bousquet *et al.*, 2004; Roche, 2006), and a remarkably high incidence of early embryonic mortality (Mann and Lamming, 2001; Bilodeau-Goeseels and Kastelic, 2003). How sub-optimal metabolism or nutrition in the dairy cow can affect oocyte and embryo quality has been reviewed extensively (Leroy *et al.*, 2008b, c, 2011). To summarize some excellent epidemiological research (Santos *et al.*, 2009; Dubuc *et al.*, 2012), it may be concluded that compromised metabolic health of the dairy cow during the transition period is associated with impaired reproductive outcome, in terms of anovulation or embryo mortality.

A number of strategies have been proposed to tackle impaired reproductive performance through an improvement of the metabolic health status of the animal. Nutrition is one of critical importance and several concepts for feeding towards an 'optimal fertility' have been proposed (Santos *et al.*, 2010; Thatcher *et al.*, 2011). One of these so-called promising strategies is feeding of fatty acids (FFA) and sources of triacylglycerols (TAG). However, without definition, this is a broad-brush approach, which could have the very opposite effect to that intended. It is vital to consider that there are different types of FFA which can be provided in varying amounts and ratios during a number of sensitive time periods. Depending of the type of fat feeding, direct effects at the level of the uterus, corpus luteum, follicle, oocyte or embryo can be expected, as well as indirect effects mediated by changes in energy balance or immune function which will ultimately impact on reproductive physiology. Therefore, comparing studies is difficult and may explain the often conflicting results in the literature. In this review, we will consider many of the key studies in an attempt to make sense of the bewildering complexity of the relationship between dietary fat and reproductive performance in dairy cows.

### Fat feeding and its effects on energy balance

Striving for an optimal metabolic health is the best strategy to safeguard normal ovarian physiology and good oocyte and embryo quality. Modern dairy rations are often supplemented with rumen protected fat to increase the energy intake in the early postpartum period and to increase fertility (Beam and Butler, 1997; Thatcher *et al.*, 2006). Dietary lipid supplementation provided to improve energy balance (DeFrain *et al.*, 2005), increases the overall dietary energy content, which stimulates milk production. An unintended downstream consequence of this increased milk production is net energy loss, ultimately resulting in elevated levels non-esterified fatty acids (NEFA) and

beta-hydroxybutyric acid ( $\beta$ -OHB) and lower concentrations of glucose and insulin (McNamara *et al.*, 2003; van Knegsel *et al.*, 2005; Moallem *et al.*, 2007). In a recent study with isocaloric diets, Van Knegsel *et al.* (2007) found out that lipogenic diets resulted in a higher energy partitioning to milk production. In particular, saturated FFA seem to induce a state of peripheral insulin resistance, increasing the amount of glucose available for lactose synthesis and thus for milk production, which further stimulates peripheral lipid mobilization (Pires *et al.*, 2007); a self-perpetuating cycle. The reported positive effects of dietary lipid supplementation on milk production depend on the precise timing of provision, with the most positive results obtained when lipids are provided as the animal reaches positive energy balance (Grummer, 1995). Together, these data suggest that supplying dietary lipids during the early postpartum period to ameliorate the negative energy balance is of little benefit to overall reproductive outcome. Indeed, pressure on metabolic health tends to increase further. Only glucogenic diets are able to alleviate the adverse effects of negative energy balance on reproductive outcome.

Recently, there has been interest on the benefits of lipid feeding during the final weeks of the dry period, in an attempt to stimulate the fatty acid provision and metabolism in the liver. Feeding lipids to the dry cow induces a rise of NEFA prepartum, but is associated with lower NEFA and lower liver TAG after calving. Researchers claim that the liver is primed to cope with FFA when presence in excess during an episode of significant lipid mobilization. However, caution is warranted as dietary fat significantly reduces dry matter intake during the dry period, which may explain part of the observations (Douglas *et al.*, 2006; Andersen *et al.*, 2008).

Another dietary strategy to minimize negative energy balance postpartum is the induction of milk TAG content depression in order to significantly reduce the energy output, since the synthesis of TAG has a high demand for energy (Bauman *et al.*, 2008). It is currently well known that several rumen fatty acid biohydrogenation intermediates (such as trans mono unsaturated FFA and conjugated linoleic acids) induce a significant drop in de novo synthesized fatty acids in the mammary gland. It has been proposed that sparing fatty acid precursors ( $\beta$ -OHB and acetate) and NADPH (made from glucose in the pentose phosphate pathway), might be of benefit to the cow. Indeed, Oden *et al.* (2007) and Castaneda-Gutierrez *et al.* (2007) demonstrated that feeding trans 10, cis 12 CLA induced MFD which was paralleled with lower NEFA and higher IGF-I concentrations and thus an improved energetic status. However, many other studies could not find any beneficial effect of the induced MFD on energy balance. We recently showed that feeding marine algae, which is rich in long chain omega-3 (n-3) FFA, caused a drop in milk fatcontent, but no beneficial effects could



be seen on energy balance. The concomitant milk yield increase suggests that at least part of the spared energy is used to stimulate milk production (Hostens *et al.*, 2011).

In conclusion, it can be stated that feeding FFA, irrespective of the fatty acid type, is not a good strategy to improve the dairy cow's energy balance. Robust scientific evidence is lacking and study results lack any consensus. Lipid feeding during the transition period can significantly reduce dry matter (DM) intake and stimulate milk yield, further aggravating the metabolic pressure on the animal.

#### **Lipid feeding as a strategy to stimulate ovarian activity and follicular growth and to alter the uterine environment**

Wathes and co-workers provided a comprehensive review of the different pathways on how dietary FFA can affect different aspects of reproduction (Wathes *et al.*, 2007). Supplemental dietary lipids increase the size of the preovulatory follicle and its production of estradiol (Lucy *et al.*, 1991; Beam and Butler, 1997; Moallem *et al.*, 2007; Zachut *et al.*, 2008), most likely via the induction of high cholesterol concentrations in follicular fluid and plasma. This increased follicle size may have beneficial effects on both oocyte quality and corpus luteum function (Vasconcelos *et al.*, 2001). The resulting hypercholesterolemia also enhances progesterone secretion, thus, supporting early embryo developmental competence (Ryan *et al.*, 1992; Lammoglia *et al.*, 1996; McNamara *et al.*, 2003). It is generally accepted that the nutritional requirements for early resumption of ovarian activity and follicular growth are different from the nutritional conditions optimal for conception and early embryo growth. In that light, Garnsworthy *et al.* (2008) advised not to increase the lipid content over 5% of the DM to avoid a depression in circulating insulin concentrations during the first weeks postpartum. However, they deliberately added dietary lipids to attenuate insulin concentrations during breeding in order to avoid oocyte and zygote overstimulation (Garnsworthy *et al.*, 2009). Apart from rations supplemented merely with saturated or monounsaturated FFA (to increase energy intake), polyunsaturated FFA (PUFA) are becoming increasingly popular; particularly as a way to increase milk concentrations of n-3 FFA and lipids containing n-3 fatty acyl residues. Supplementation with these polyunsaturated FFA can suppress prostaglandin secretion by the endometrium, and hence support the lifespan of the CL (Staples *et al.*, 1998; Cheng *et al.*, 2001; Thatcher *et al.*, 2006), an effect which would be beneficial for embryo survival. The mechanism behind this observation was reported by Bilby *et al.* (2006b) who showed that diets rich in fish oil (high in n-3 polyunsaturated) have the potential to reduce the expression

of endometrial cyclooxygenase-2, an essential enzyme for prostaglandin biosynthesis (Thatcher *et al.*, 2006). In stark contrast, Hinckley *et al.* (1996), demonstrated that fish oil inhibited progesterone production by luteal cells cultured *in vitro*. This observation was confirmed *in vivo* by a study in which cows were fed a linseed rich diet (linolenic acid, C:D18:3, n-3) which led to significantly reduced plasma progesterone concentrations (Robinson *et al.*, 2002). Mattos *et al.* (2002) were not able to corroborate this negative effect on progesterone production in synchronized cows fed either eicosapentaenoic acid (EPA, C:D 20:5, n-3) or docosahexaenoic acid (DHA, C:D 22:6, n-3). In other words, it is important to consider the exact type of the supplemented FFA (length of the carbon chain and degree of unsaturation) when estimating a specific effect on fertility. Feeding n-6 FFA to dairy cows stimulates PGF2- $\alpha$  synthesis improving uterine health (Petit *et al.*, 2004). A sequential and selective feeding of extra n-6 FFA around calving and of n-3 rich diets during the breeding period has therefore been proposed as an optimal reproductive management strategy in dairy cows (Silvestre *et al.*, 2011). The optimal immune response at the uterine level early postpartum should prevent endometritis while the n-3 supplementations around conception should safeguard embryo survival through sustained corpus luteum function. Clearly, a conclusive result of the effects of fat supplementation in dairy rations on the reproductive outcome, awaits further investigation.

#### **Lipid feeding and the effects on the oocyte and embryo microenvironment**

It is widely reported that changes in serum FFA are reflected in the lipid composition of the follicular environment (Childs *et al.*, 2008b; Fouladi-Nashta *et al.*, 2009). For example, PUFA content in follicular fluid is highly correlated to that of the diet (Adamiak *et al.*, 2005) and it is generally accepted that alterations in dietary fatty acid intake cause a similar shift in the fatty acid profile of the follicular fluid (Wonnacott *et al.*, 2010; Zachut *et al.*, 2010) although the ovary can, to some extent, buffer against major fluctuations in plasma n-3 and n-6 fatty acids (Fouladi-Nashta *et al.*, 2009).

One of the best-studied examples of metabolic changes in the follicle fluid is the phenomenon of NEB in high-yielding dairy cows (Leroy *et al.*, 2008a). In summary, there is good evidence that the ovary can selectively accumulate NEFA in a way that means that the concentration of FFA in plasma correlates to that measured in follicular fluid (Canfield *et al.*, 1990; Grummer *et al.*, 1995; Rabiee *et al.*, 1997; Comin *et al.*, 2002; Leroy *et al.*, 2005). Similar correlations between plasma and follicular fatty acid composition have recently been reported in humans (Robker *et al.*, 2009; Valckx *et al.*, 2012). Interestingly, palmitic acid, stearic acid, and oleic acid are the predominant NEFA in



bovine (Leroy *et al.*, 2005) and human ovarian follicle (Valckx *et al.*, 2012).

Data concerning the microenvironment within the oviduct and uterus are less well established due to technical difficulties in sampling the environment. Leese *et al.* (2008) proposed the epithelia lining the endosalpinx and endometrium as the final components in a supply line that links maternal diet at one end and embryo uptake of nutrients at the other. Also Tsujii *et al.* (2009) emphasized that serum and oviduct fluids play an important role in the development of blastocysts. The concentrations of nutrients in tubal fluid are documented to be below their plasma concentrations (Leese and Barton, 1984), which suggests that their overall transport across the tube occurs principally by diffusion (Leese and Gray, 1985); however, there are ongoing reintensified efforts to attempt to model transport of nutrients into the female reproductive tract. It is clear from the work of Childs *et al.* (2008a) that PUFA feeding affects the fatty acid composition of the genital tract.

#### The influence of fat feeding on the oocytes and embryonic lipid profile

Although the fatty acid composition of oocytes across a number of mammalian species has been reported (McEvoy *et al.*, 2000), little is known about the uptake of specific FFA by the follicle enclosed oocyte, how this may be altered by maternal metabolism and the consequences this might have for postfertilization development. A number of *in vitro* studies from different species and using diverse approaches have shown that the lipid profile of oocytes is dynamic and can be influenced by the external environment (Ferguson and Leese, 1999; Sata *et al.*, 1999; Kim *et al.*, 2001; Adamiak *et al.*, 2005; Aardema *et al.*, 2011). The lipids stored within the oocyte and early embryo represents an important source of energy for the early embryo (Sturmey *et al.*, 2009; McKeegan and Sturmey, 2012), however the consequences of endogenous lipids on early development have historically been overlooked. Oocytes have been shown to have increased TAG content when cultured with 'lipid enriched' follicular fluid, leading to compromised nuclear maturation (Yang *et al.*, 2012). In the broadest physiological terms, unsaturated fatty acids tends to have beneficial effects, whereas saturated fatty acids tend to have more deleterious effects. This is largely borne out in the oocyte and early embryo. For example, human embryos containing a higher ratio of unsaturated to saturated fatty acids are more likely to progress beyond the 4 cell stage (Haggarty *et al.*, 2006). What is less clear is the extent to which lipid composition of the oocyte and embryo *in vivo* can be altered in response to diet (Zeron *et al.*, 2002) and whether this impacts embryo quality. Santos *et al.* (2008) suggested the existence of a selective uptake process to ensure that the

PUFA content of oocytes is kept to a minimum to minimize risks for degradation. Also Fouladi-Nashta *et al.* (2009) proposed that the ovary can buffer the oocyte against major fluctuations in plasma PUFA. In embryos, a similar protection mechanism might exist, as they found higher concentrations of saturated fatty acids than unsaturated fatty acids in rabbit embryos (Tsujii *et al.*, 2009).

#### Fat feeding and the effects on oocyte and embryo quality

As discussed earlier, the period of follicular development and early embryo development may represent a 'window of susceptibility' to dietary induced changes in the maternal environment (Ashworth *et al.*, 2009). In cows, supplementation with linoleic and linolenic acid, as present in sunflower and linseed oils, has little effect on *in vitro* maturation, subsequent oocyte quality, fertilization, or embryo development (Bilby *et al.*, 2006a). Feeding ewes with fish oil supplemented diet improved oocyte quality, oocyte membrane integrity, and increased the proportion of PUFA in the plasma, follicular fluid, and cumulus cells, but not in the oocyte (Zeron *et al.*, 2002). A diet with high n-3:n-6 ratio has been shown to increase linolenic acid and estradiol levels in the follicle and improve embryo cleavage rate (Zachut *et al.*, 2010), and conjugated linoleic acids, decrease embryo development rate and also suppress expression of stearoyl-CoA desaturase-1, the enzyme which converts stearic acid to oleic acid (Stinshoff *et al.*, 2013). A very recent study done in Brazil could not show any positive effects of supplementing linoleic acid to the diet of Nellore heifers on embryo production. On the contrary, embryo cryotolerance was significantly reduced in the fat supplemented group (Guardieiro *et al.*, 2013). Dietary intakes of women in the month preceding *in vitro* fertilization or intracytoplasmic sperm injection treatment showed that a high n-3 intake was associated with improved embryo morphology (Hammiche *et al.*, 2011). Confusingly however, high maternal dietary n-3 PUFA supplementation periconception reduced normal oocyte development in the mouse, perturbed mitochondrial metabolism, and adversely affected the morphological appearance of the embryo (Wakefield *et al.*, 2008). Furthermore Petit *et al.* (2008) reported that feeding flaxseed as a source of alpha-linolenic acid (ALA) did not improve embryo quality or the maintenance of gestation after embryo transfer.

Combined, the studies described in the preceding paragraph, as well as a great number of other important studies, illustrate the complexity of the relationship between nutrition and oocyte/embryo quality. For an overview of some recent studies about the effects of dietary lipid supplementation on oocyte and embryo quality, see Table 1. When designing and evaluating studies in this area, careful consideration



must be given to the precise timing and duration of dietary intervention, as well as to the amount and chemical nature of the lipid supplement. It is also important to note that there may be species-specific response to dietary lipid supplementation in terms of oocyte and embryo quality and care must be taken when extrapolating from mouse models. Furthermore, studies often identify the level (oocyte and/or embryo) where the dietary induced lipid changes impact on fertility, though they do not distinguish the specific lipid fraction and its structural composition responsible for observed effects. We have previously reported that exposure of

preimplantation embryos to dietary-induced hyperlipidemic serum can result in reduced embryo development and quality, hence poorer fertility (Leroy *et al.*, 2010). The mechanistic insights for these findings are lacking so far, as the supplemented sera contained several lipid fractions that were significantly altered in response to the dietary lipid supplements, including doubled cholesterol concentrations, more than doubled total fatty acid concentrations, and increased levels of both long chain saturated and unsaturated fatty acids (Leroy *et al.*, 2010). Here, *in vitro* studies become invaluable.

Table 1. Survey of studies focusing on the effect of different types of fatty acids on oocyte and embryo quality in ruminants.

Author	Findings
Aardema <i>et al.</i> , 2011	<b>Oleic acid</b> rescues effects of palmitate and stearate and promotes maturation and development.
Adamiak <i>et al.</i> , 2006	<b>Altered lipid intake</b> is reflected in changed fatty acid composition in follicular fluid and cumulus oocyte complex.
Bilby <i>et al.</i> , 2006	Negative effects of <b>n-6 rich</b> diets on oocyte quality.
Chankitisakul <i>et al.</i> , 2013	<b>L-carnitine</b> treatment dislocates lipid droplets and improved cryopreservation of bovine oocytes.
Fouladi-Nashta <i>et al.</i> , 2007	Positive effect of 800 g <b>Megalac®</b> supplementation for 14 days on oocyte quality.
Fouladi-Nashta <i>et al.</i> , 2009	Holstein cows fed <b>palmitic</b> and <b>oleic, linoleic</b> or <b>linolenic acids</b> had altered plasma fatty acid profile, but no effect on embryo development rate.
Haggarty <i>et al.</i> , 2006	Human embryos with higher <b>unsaturated:saturated fatty acid ratios</b> are more likely to develop.
Hughes <i>et al.</i> , 2011	<b>EPA and DHA</b> may increase oxidative damage in ovine oocytes.
Junghheim <i>et al.</i> , 2011	Predominant human follicular fluid and serum NEFA were oleic, palmitic, linoleic, and stearic acid. Elevated NEFA correlated with poor COC morphology
Lapa <i>et al.</i> , 2011	Improved development and embryo quality after <b>trans-10 cis-12 CLA</b> supplementation during bovine oocyte maturation.
Marei <i>et al.</i> , 2009	Positive effect of <b>linolenic acid</b> on oocyte <i>in vitro</i> maturation.
Marei <i>et al.</i> , 2010	Negative effects of <b>linoleic acid</b> on oocyte <i>in vitro</i> maturation and developmental potential.
Oba <i>et al.</i> , 2013	High concentration of <b>NEFA</b> <i>in vivo</i> derived serum might adversely affect early cleavage stages in bovine embryos.
Ponter <i>et al.</i> , 2012	Bovine diet high in <b>linolenic acid</b> increases Prostaglandin E2 synthase-1 expression in COCs.
Yang <i>et al.</i> , 2012	<b>Lipid-rich human follicular fluid</b> decreases murine oocyte maturation rate.
Zachut <i>et al.</i> , 2010	Better cleavage rate after <i>in vitro</i> fertilization of oocytes from <b>linolenic acid</b> supplemented cows.
Zachut <i>et al.</i> , 2010	Bovine diet with a high n-3:n-6 ratio increases <b>linolenic acid</b> and estradiol concentrations in the follicle.
Zeron <i>et al.</i> , 2002	Positive effects of <b>fish oil</b> supplemented diets on oocyte quality and chilling sensitivity.

#### Use of *in vitro* models to understand oocyte and embryo responses to 'fat'

By using *in vitro* models, it is possible to assess the direct effects of individual and combinations of FFA on oocyte and early embryo development in a controlled way. This research has been reviewed previously (Sturmey *et al.*, 2009; McKeegan and Sturmey, 2012). Whilst care must be taken when extrapolating such studies to the whole animal, *in vitro* studies have given

us a wealth of understanding on how specific lipid molecules can impact early development. For example, addition of physiological concentrations of n-3 PUFA to oocyte maturation media resulted in improved oocyte nuclear maturation rate, whereas n-6 PUFA-treated oocytes had reduced resumption of meiosis (Marei *et al.*, 2009, 2010). In bovine oocytes, n-3 PUFA may play a critical role in maintaining meiotic arrest (Homa and Brown, 1992), possibly acting through protein kinase C (Murakami *et al.*, 1986), which plays a significant role in



metabolic regulation on a cellular level, in cell growth, and differentiation (Nishizuka, 1988).

What is especially interesting are emerging data showing that the phenotype of the early embryo can be dramatically altered by exposure to FFA during the final oocyte maturation. Bovine *in vitro* maturation models have demonstrated that elevated concentrations of saturated NEFAs, such as stearic acid and palmitic acid, can reduce oocyte developmental competence (Jorritsma *et al.*, 2004; Leroy *et al.*, 2005; Aardema *et al.*, 2011). We have recently shown that exposure to elevated NEFA during oocyte maturation can lead to profound changes in metabolic regulation and gene expression in the resulting embryo (Van Hoeck *et al.*, 2011, 2013). Of particular note was the observation that oocyte exposure to elevated NEFA lead to embryos which, at the blastocyst stage, did not consume glucose. This is a startling observation, since a sharp increase in glucose consumption at the blastocyst stage is common to all species studied thus far (Smith and Sturmeijer, 2013). The impact of this metabolic deregulation in the early embryo is currently unclear, but does suggest that the period when follicles are developing may represent a 'window of susceptibility' to the dietary or metabolically induced differences in fatty acid availability with the consequences persisting in the embryo. Too high fatty acid provision in the oocyte's microenvironment due to massive lipolysis (negative energy balance) or due to specific fat feeding strategies may lead to reduced fertility due to compromised early embryo quality. Much more research, is needed to define optimal dietary lipid supplementation strategies. And finally, enough attention should be paid to unforeseen potential negative effects of dietary lipid supplementation having indirect negative effects on reproduction. To give one example, Wullepit *et al.* (2012) recently demonstrated that PUFA feeding to dairy cows significantly increased the level of oxidative stress.

### Conclusions

It can be concluded from this overview that dietary lipid supplementation has very limited additive value in alleviating the negative energy balance during the transition period. Dietary lipid supplementation can be a good strategy to stimulate follicular growth and steroid production. Depending on the source of lipids given, the effect on prostaglandin synthesis in the uterus or corpus luteum can be very different. Direct effects on oocyte and embryo quality tend to vary significantly and the results may depend on experimental set up and the animal model used. More *in vitro* studies are warranted to provide us with in depth knowledge on the pathways involved. Finally it is important to consider the more indirect effects of dietary lipid supplementation on reproduction, for example due to an altered dry matter intake, ruminal health, immunity, oxidative stress, and endocrine signalling.

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## Sperm biotechnologies in domestic species: state of the art

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### Abstract

Modern livestock breeding is basically dependent on the proper use of semen for artificial insemination (AI) of females and of other reproductive biotechnologies such as the production of embryos in vitro for embryo transfer (IVP). Both these techniques have made possible not only the wide dissemination of genetic material onto breeding populations but also enhanced the selection of best sires, owing to the development of better diagnostic techniques for sperm function and of preservation of seminal material over time. Although use of liquid semen cooled to room temperature, to intermediate temperatures (+16-20°C) or chilled (+5°C) dominates in different species, cryopreservation is preferred in bovine AI and it is advancing in other species by the design of new containers, freezing methods and the use of better insemination strategies. Techniques to separate the aliquot of most robust spermatozoa from an ejaculate have shown a reascent particularly for sires with low sperm quality, and technological advances in separating spermatozoa for chromosomal sex make the technique suitable for commercial use, following application of novel findings in sperm and seminal plasma (SP) diagnostics and function. Alongside, knowledge of the epigenome and signalling capabilities of the semen (sperm and SP) calls for further studies regarding transgene production via ICSI for IVP or AI.

**Keywords:** AI, cryobiology, IVF, selection, semen, sexing.

### Introduction

Generations of reproductive biotechnologies have been developed as routine applications to safely propagate genetic material among breeding populations. Moreover, they intend to shorten generational intervals and to focused selection of specific traits and production of specific genders. In some of these technologies such as the predominant AI and in IVP for embryo transfer, focus is on spermatozoa and recently on SP. Furthermore, new challenges in cryobiology, sperm desiccation, sperm selection and sperm survival to manipulations have arose, particularly in species where selection for sperm quality has not been primarily

addressed. Semen is still the “cheapest” component of artificial breeding, something that explains the dominance of AI over any other reproductive biotechnology. AI is, alongside its sanitary advantage, the best technique to issue the large dissemination of desirable genetic characters on a female population, propagating the genetic material of selected stud sires, which are continuously replacing the best ones we presently use (Rodríguez-Martínez, 2012a). Successful freezing of semen of all livestock is a long lasting priority, tied not only to acceptable cryosurvival and lifespan after thawing but also to the devise of rational techniques that can provide largest possible numbers of doses for AI at the lowest possible cost. Last but not least, the deposition of these doses is to be easy and yield acceptable fertility, i.e. close to use of cooled semen or even natural mating. Unfortunately, a better knowledge of the moment of spontaneous ovulation is mandatory, demanding a holistic approach to the technology of semen processing and use.

The present review summarizes the state-of-the-art in some prevailing semen biotechnologies such as sperm (cryo)preservation for use in AI or IVF, focusing on low-sperm number AI-doses, and spermatozoa subjected to sperm selection techniques including sex-sorting via flow cytometry or other techniques. Particular attention is taken on the feasibility of these techniques for commercial application in view of the high susceptibility of spermatozoa to be modified or succumb alongside the treatments. Reascent manipulations such as alternative preservation methods or their use as DNA/RNA adsorbed vectors for transgene production is also included.

### Sperm preservation

Semen preservation has historical roots that document back to the 18th century, with a boom experienced during the first half of the 20th century in relation to the development of AI with liquid semen. From the 1950s, the application of cryoprotectants contributed to the wider use of semen freezing, particularly for the application of intrauterine AI in dairy cattle. During the past 40 years, the development and use of AI with preserved semen have grown exponentially and on a global scale, particularly in the breeding of dairy cattle (>200 million of the first AIs in

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the world use frozen semen) and pigs (>160 million used cooled liquid semen doses). In Europe, the Americas and South-east Asia, sows are basically bred via AI with liquid (non-frozen) semen, mimicking the situation already reached in dairy cattle. Cattle semen is cryopreserved using standardized methods for extension, cooling, freezing and thawing basically all over the world, with only subtle differences between *B. Taurus*, *B. indicus*, *Bubalus bubalis* or *Bos javanicus* (Rodríguez-Martínez, 2007a; Rodríguez-Martínez and Barth, 2007).

However, the current available methods to preserve semen as a genetic resource and its successful dissemination via AI and other assisted reproductive technologies (ARTs) are still sub-optimal (Rodríguez-Martínez, 2012a, c). This is a fact even in dairy cattle, visible when sperm numbers per dose are lowered after sex sorting, and survival of potentially fertile spermatozoa reaches a threshold and fertility does not reach values higher than 60-70% of the fertility of the conventional (non-sexed) semen (Rodríguez-Martínez, 2012a, c). Other species, such as porcine, equine, canine and ovine, seem condemned to using extended chilled liquid semen despite its limited shelf-life, its decline in fertility over transit time, and risks of damage due to temperature, pressure or handling changes. While use of liquid-chilled semen has grown exponentially over the past decades, use of frozen semen is restricted for these species to 1-3% of total AIs worldwide (Rota *et al.*, 1997; Gil *et al.*, 2003; Riesenbeck, 2011). Semen is still being “best” cryopreserved using slow-freezing protocols originally devised in the mid 1960-1970’s with empirical modifications introduced over time (Katkov, 2012; Morris *et al.*, 2012; Rodríguez-Martínez, 2012b). In general, extension is done using egg yolk or milk-containing media in most livestock following the extension or removal of most of the SP. The freezing media most often includes glycerol as cryoprotectant (CPA) and in some species a surfactant (often laurylsulphate, Orvus es Paste-OEP) is also included. Spermatozoa are cooled beyond the eutectic temperature at 30 to 50°C/min and thawing is done at 1,000-1,800°C/min (see Katkov, 2012). The entire freezing procedure takes up to 8-9 h from semen collection to storage of the frozen doses in LN<sub>2</sub>, and for some species yields few AI-doses per ejaculate (Rodríguez-Martínez, 2012b). Variation is still present between ejaculates and among males for their capacity to survive cryopreservation. Often, cryopreservation protocols have to be modified to accommodate males with suboptimal sperm freezability (so-called bad freezers), particularly regarding glycerol concentration and warming rates (Knox, 2011). Those changes usually allow for minimum acceptable cryosurvival (i.e. between 30 and 40%), confirming its suboptimality. Moreover, the weakening of the surviving cells (lifespan, deteriorated attributes, fertilizing capacity, genome damage), leads to lowered fertility (Rodríguez-Martínez, 2012a), which implies we are far from reaching the goals set up by the

industry for frozen-thawed semen: >80% conception rates and, for pigs, a litter size of 11 piglets (Knox, 2011). This scenario limits most frozen semen to research, genetic banking or the export of semen for selected nuclei lines, impairing its wider use. Publications in species with clearly suboptimal freezing capacity increased from 18 in 1980 to 2,103 in 2012 (Pubmed <http://www.ncbi.nlm.nih.gov/pubmed/>. Access on April 12, 2013), indicating the solution is yet to come. Obviously new cryopreservation methods ought to be developed.

#### *Advances in sperm cryobiology?*

Over the past decade, the cryobiology of semen has diminished its empiric approach towards (i) the determination of *in vivo* features (particularly regarding SP-composition), (ii) the action of specific additives and different CPA, (iii) the use of automated freezers and of directional gradient freezing and, (iv) the freezing of lower-sperm numbers/dose owing to intrauterine AI or use of sexed semen (Rodríguez-Martínez, 2012c). Yet, there is an intrinsic male-to-male variation that needs clarification. Methods for cryopreservation have not changed much. Spermatozoa are frozen slowly, with extracellular ice formation, dehydration, a toxic hyper-concentration of intracellular solutes which does not resolve during thawing, and jeopardizing cell survival or affecting vital cell functions post-thaw (Rota *et al.*, 1997; Gil *et al.*, 2003; Saragusty *et al.*, 2009; Morillo-Rodríguez *et al.*, 2011; Rodríguez-Martínez, 2012b). Solute-caused damage can be minimized by CPAs (such as glycerol, dimethyl sulphoxide [DMSO], ethylenglycol [EG], propyleneglycol [PG]) all highly soluble, permeating compounds of low-to-medium toxicity at low concentrations. They increase the total concentration of all solutes in the system, thus reducing the amount of ice formed at any given sub-zero temperature; *slow equilibrium freezing* (Pegg, 2007; Saragusty and Arav, 2011). With sufficient CPA introduced, high cooling rates (i.e. dipping into LN<sub>2</sub>) would eventually solidify the sperm suspension into a metastable glassy, vitreous state with no ice formed; rapid, non-equilibrium *vitrification* (Pegg, 2007; Saragusty and Arav, 2011). Ultra-high rates void the need of toxic penetrating CPA and opens for non-penetrating CPA (such as sucrose or trehalose). Use of these CPAs has made possible the vitrification of dog, human and rabbit spermatozoa, yet with low survival (Sánchez *et al.*, 2011; Isachenko *et al.*, 2012; Rosato and Iaffaldano, 2013). Ultra-high cooling rates (10,000 °C/min; Rodríguez-Martínez, 2012b) are seen a major prerequisite for improvement (Arav and Natan, 2009; Saragusty and Arav, 2011).

#### *Basic research opens new possibilities*

In species with fractionated ejaculation (canine, porcine, equine, human) spermatozoa are mainly



ejaculated in SP with specific SP-proteins (Rodríguez-Martínez *et al.*, 2011) and anti-oxidant enzymes, such as paraxonase-1 (PON1, Verit *et al.*, 2009), which effectively sustain sperm survival. Although ejaculated spermatozoa are an heterogeneous population, they certainly share basic concepts: (i) a sperm head with substantially less free water than the neck and tail segments (Morris *et al.*, 2012), (ii) consistent use of anaerobic glycolysis alongside aerobic respiration (Silva and Gadella, 2006), (iii) a membrane with water channels including glycerol-transporting aquaglyceroporins (Ekwall, 2007), (iv) plasmalemmal propensity to lipoperoxidation (LPO) by exposure to reactive oxygen species (ROS; Ortega Ferrusola *et al.*, 2009), which (v) facilitates for oxidative DNA damage under sperm storage (Aitken and Koppers, 2011). Stem cells (Darzynkiewicz and Balazs, 2012), appear often in niches surrounded by low O<sub>2</sub> levels (pO<sub>2</sub> 1-6%, against 2-9% in most tissues or 21% in air). Such “hypoxia” levels prevent cell differentiation (Eliasson and Jönsson, 2010). Hyaluronan (HA, the *in vivo* existing polyanionic form of hyaluronic acid) an abundant, ubiquitous, non-antigenic glycosaminoglycan, well-conserved over taxa (Volpi *et al.*, 2009) is also present in these niches. By comparison, the low pH, low bicarbonate and low O<sub>2</sub> conditions of the epididymal caudae preserves sperm survival and fertilizing capacity (Rodríguez-Martínez *et al.*, 1990; Rodríguez-Martínez, 1991), as when lowering extender pH (Lafluf *et al.*, 1990; Rodríguez *et al.*, 1994). The same conditions seem to be present in the oviductal sperm reservoir (Rodríguez-Martínez, 2007b; Preston and Sherman, 2011) with HA being conspicuous in pigs and cows (Rodríguez-Martínez, 2001) preventing sperm capacitation and acrosome exocytosis (Tienthai *et al.*, 2004; Bergqvist *et al.*, 2005). HA has been reported as protecting cells from ROS-induced DNA damage by chelating Fe<sup>2+</sup> and Cu<sup>2+</sup> ions that contribute to formation of OH-radicals (Balogh *et al.*, 2003). Spermatozoa express HA receptors on the cell surface, apparently with specific roles for maturation, motility and fertilization (Tienthai *et al.*, 2003). HA favors preservation of stemness during long-term culture of embryonic stem cells (Ramírez *et al.*, 2011) and embryo development in several species (Gardner *et al.*, 1999; Suzuki *et al.*, 2002; Palasz *et al.*, 2006). Considering the above and the fact that HA has proven a good CPA for somatic cells (Ujihira *et al.*, 2010) and beneficial for boar sperm cryosurvival (Peña *et al.*, 2004), a combination of relative low O<sub>2</sub> levels (hypoxia), HA and specific SP-components ought to be tested while preserving spermatozoa via vitrification.

#### *MicroRNAs, not to be forgotten...*

Semen contains spermatozoa with a nuclear genome that has to be intact to participate in embryo development, so DNA is explored for intactness when sperm is manipulated and cryopreserved (see above).

Semen also delivers a series of small regulatory non-coding RNAs (ncRNA, 19-22 nucleotides; Bartel, 2009), microRNAs (miRNAs) shed both in the SP (Belleannee *et al.*, 2012; Wu *et al.*, 2012) and also present within each spermatozoon (Hamatani, 2012; McIver *et al.*, 2012). These miRNAs are key post-transcriptional modifiers of gene expression, e.g acting epigenetically, and play an important role in the acquisition and maintenance of male fertility (Dadoune, 2009). Numerous in bull sperm, they show differential expression in relation to fertility levels of the sires (Govindaraju *et al.*, 2012). Delivered to the oocyte at fertilization, they modulate first cleavage divisions (Liu *et al.*, 2012). In freeze-resistant insects but also vertebrates, they are responsive to freezing, and may act to rapidly regulate metabolic responses to survive freezing stress (Biggar *et al.*, 2009).

#### *But, does cryopreservation cause epigenetic alterations in chromatin structure?*

Reports are controversial. Suboptimal cryopreservation leads to alterations in chromatin structure, often in relation to cell viability restrictions in spermatozoa of several species (Rodríguez-Martínez, 2012b), in human oocytes (Monzo, 2012) or in murine blastocysts (Larman *et al.*, 2011). Significant down regulation of house-keeping and function related genes have been seen (Karimi-Busheri *et al.*, 2013), as well as changes in transcriptomes in embryonic stem cells (Wahg *et al.*, 2011), while opposite reports are also available regarding mesenchymal stem cells (Angelo *et al.*, 2012). Hence, there is a large need to resolve how different methods of cryopreservation impact the epigenetic fingerprint of spermatozoa.

#### **Low-sperm numbers in AI doses: the prevailing trend**

Despite the above exposure, preservation of semen for AI is routine providing semen of good quality for AI on commercial basis, despite using excessive sperm numbers per AI-dose. The reason behind is the low cryosurvival we experience (most often less than 50% of the spermatozoa survive) and, despite we still struggle in trying to understand how spermatozoa lose their capacity to remain fertile upon freezing and thawing, many of the survivors have changed attributes, among which a shortened life-span is predominant. There is an overall tendency among AI-semen cattle producers to reduce sperm numbers per AI-dose. Several reasons are behind this trend; (i) to increase revenues, (ii) to select sires by their innate optimal fertility level and, (iv) to accommodate the increasing use of sex-sorted semen. While spermatozoa from most sires survive cryopreservation using current protocols, some others do not, for reasons not yet fully understood. More crucial is the freezing of flow-cytometrically sex-sorted



spermatozoa, whose membrane is clearly affected by the processing and thus require of particular modifications in the freezing protocols (Parrilla *et al.*, 2012; Balao da Silva *et al.*, 2013) including the restoration of SP (de Graaf *et al.*, 2008) or of specific SP-proteins (Caballero *et al.*, 2012). The AI of low-sperm doses, including those containing sex-sorted semen by flow cytometry (Garner and Seidel, 2008), is usually carried out more or less deep into the uterine horn with acceptable results in bovine (Ballester *et al.*, 2007; Seidel and Schenk, 2008; Schenk *et al.*, 2009) or porcine using the deep-intrauterine AI procedure (Roca *et al.*, 2011) specially when using frozen-thawed boar semen or sex-sorted semen effective by properly trained deep AI (Wongtawan *et al.*, 2006; Roca *et al.*, 2011).

### Sperm selection

Sperm selection is a wide term, comprising methods for separation of spermatozoa for *in vitro* fertilization (IVF), for sperm size, for membrane and DNA intactness, for motility, or for the enrichment of the population of spermatozoa bearing either an X- or a Y-sexual chromosome (sex-sorting). For IVF, washing by extension and centrifugation, filtration/gradient separation or self-motility (the so-called swim-up or -down) techniques are most common (Rodríguez-Martínez *et al.*, 1997). The grade of enrichment of the most robust spermatozoa from the ejaculate (i.e. the effectiveness of the method) depends, however, on the sperm numbers present and whether the sample contains a high proportion of abnormal spermatozoa. Neither case is common among livestock ejaculates. Some techniques are, however, used for enrichment after thawing, where the amount of surviving spermatozoa is low and there is an interest to remove dead, moribund and abnormal spermatozoa. Most farm animal spermatozoa in a normal semen sample show a typical progressive, linear motility. Spermatozoa use this innate linearity to convey natural fluid barriers such as the cervix or the uterine-oviductal junction and thus it has been related to fertility in farm animals, thus leading to methods that mimic *in vivo* situations, as swim-up. By overlaying a semen sample with an appropriate volume of a suitable fluid (often culture medium but also with more complex preparations of varying viscosity) followed by incubation, during which time the motile spermatozoa migrate actively into the overlay medium, this simple procedure selects for sperm motility and membrane integrity, essential parameters for fertilization. Sperm numbers and their speed when traversing the column might define the most fertile sample. The method separates a sperm sub-population with most intact attributes for further testing, such as membrane, acrosome, mitochondrial, and DNA integrity as well as a higher penetrability of the zona pellucida (ZP), and higher ability to produce blastocysts after IVF (Zhang *et al.*, 1998). Additives for the swim-up media

have proven beneficial; for instance, recovery and cleansing of high quality bulls spermatozoa are facilitated by adding homologous or heterologous cervical mucus or hyaluronan (a component of the oviductal fluid) to the swim-up medium (Shamsuddin and Rodríguez-Martínez, 1994). Another method to separate by linearity is the centrifugal counter-current distribution analysis (CCCD), an aqueous two-phase partition system, which has proven valuable for revealing sperm heterogeneity in semen samples and, indirectly, shown correlations to fertility (revised by Rodríguez-Martínez, 2007c). Novel methods have recently been developed using alternative multiple microfluidic flow streams for sperm self-migration which allow for the sorting of motile spermatozoa (Smith *et al.*, 2011; Wang *et al.*, 2011). In sum, self-migration procedures select spermatozoa as *in vivo*, but they can not isolate large sperm numbers, becoming mostly suitable for IVF.

Other methods have, therefore, put forward as substitutes where a higher output of an intact population is selected, a prerequisite when dealing with farm animals having large ejaculates. Examples of these methods are the centrifugation through columns of adherent particles (Sephadex or glass-wool, Januskauskas *et al.*, 2005) or the differential centrifugation through discontinuous density gradients of silane-coated silica spheres (Rodríguez-Martínez *et al.*, 1997). Centrifugation through a single column of species specific formulations of colloid (based on silate-coated spheres, the SLC method) has proven successful to harvest the most robust spermatozoa from any (raw or serially processed) semen suspension, in most species tested so far (Morrell and Rodríguez-Martínez, 2009, 2010; Morrell *et al.*, 2010). The selective power of the latter method is clearly related to species differences in osmolarity and density of the colloid (Morrell *et al.*, 2011), being more advantageous in terms of less damage and better recovery rate for spermatozoa with intact fertilizing attributes.

### Sperm sex-sorting

Gender selection is, in livestock production, highly desirable; heifers for dairy cattle, males for beef cattle. In pig production, it would also allow the production of either male or female crossbred lines, or ameliorate the incoming problem of the banning of male piglet castration in Europe. Using the Beltsville Sperm Sexing Technology, based on high speed flow cytometry sorting of DNA-stained spermatozoa with difference in size (and thus emitted fluorescence to a laser beam exposure) between the sex chromosomes (revised by Johnson *et al.*, 2005) the enrichment of AI-doses for either X- or Y-chromosome-bearing spermatozoa is now a commercial reality. At commercial level, doses, enriched to >90% level have resulted in the birth of >50,000 documented calves of the desired sex, with



>95% or success, and figures are increasing (Garner, 2006; Schenk and Seidel, 2008; Garner and Seidel, 2008; Seidel, 2009). Although the numbers of sorted spermatozoa per hour reach at present larger figures than a decade ago (100-200 million compared to 350000), these numbers yet imply few sperm doses produced, impairing their application for conventional AI. However the technology is facing today a strong wave of increasing commercialization in cattle (Garner and Seidel, 2008; Seidel, 2009) as well as it is becoming promising for other species (porcine, equine), including the provision opportunities for sex selection of IVP-embryos, surpassing the need for sex diagnosis of embryos (Blondin *et al.*, 2009; Carvalho *et al.*, 2010). Although the only fully validated technology for pre-selecting offspring for sex available at present is the Beltsville method, new applications are coming into the market such as use of antibodies against sex-specific proteins, which can -by immobilizing spermatozoa of a certain sex- allow for handling of the spermatozoa bearing the other (Cattle Logic Ltd, UK). Sex-sorting is however, still too costly (a flow sorter costs above US\$ 300000), slow, and still yields spermatozoa with reduced lifespan (Lonergan, 2007; Gosalvez *et al.*, 2011). Nevertheless, the products (male- or female-sorted spermatozoa) are available and becoming more competitive by the day (Hayakawa *et al.*, 2009; Underwood *et al.*, 2009, 2010).

A series of problems yet shadows the commercial application of sex-sorted boar or stallion semen for AI; firstly, the well-known low survival of the sorted spermatozoa, a matter that affects spermatozoa from all species; related to the high pressure and to the extreme sperm extension applied during the process (Vazquez *et al.*, 2009), conveying detrimental effects of the absence of SP-components (Caballero *et al.*, 2012). Secondly, sperm sexing is slow, thus inappropriate for standard pig AI, requiring deep-intrauterine AI (see above). The above mentioned drawbacks of the current technology have been compensated by the use of additives to the sperm-media (mostly as SP) and the growing application of deep intrauterine AI (Vazquez *et al.*, 2009). Freezing of sex-sorted boar spermatozoa has been tested and proven usable for IVF; the embryos obtained are capable of establishing pregnancies to term (but not carry to term) after nonsurgical ET (Bathgate *et al.*, 2007). Obviously, owing to the enormous impact that sperm-mediated gender selection would have in pig production, a further development of the flow cytometry sex-sorting technique or alternative methods are hoped for, demonstrating how far they might be from commercial use.

### Spermatozoa as vectors for transgene production

Sperm-Mediated Gene Transfer (SMGT) has been shown to be a practical method to produce

transgenic animals, without requiring embryo handling or expensive equipment (Lavitrano *et al.*, 1989). Although the mechanism governing foreign DNA integration is not well understood and yet controversial (Eghbalsaied *et al.*, 2013), SMGT has proven to be highly efficient in integrating the transgene into the genome of the pig embryo (57-80%, based on ~200 generated pigs, compared to microinjection (Lavitrano *et al.*, 2006). Transgenic pig lines produced by SMGT have been reported since 1997 (Lavitrano *et al.*, 1997, 2002, 2003) and the methodology is well established (Lavitrano *et al.*, 2013). SMGT efficiency has been shown to be increased by ICSI of spermatozoa coated with DNA (Kurome *et al.*, 2006) and proven to work in other domestic animals, such as equine (Zaniboni *et al.*, 2012) or bovine (Simões *et al.*, 2012).

### Future trends

Development of better (cryo)preservation methods for pigs are a priority for our research group. AI is a low-cost technology for commercial pig production and therefore making it interesting to maintain and select specific stud boars, similarly to what is practice in cattle. For instance, if the aim is to diminish undesirable alleles in a general population, a logical way is to start genotyping stud boars genetic markers related to these alleles and amortize such investment (and that of semen freezing) by selling the semen doses of those boars found free. The other interested party is the producer with an elite breeding herd, who is the most interested to buy semen from the genotyped boars with two good alleles for a particular gene, since they can, using AI, reduce the frequency of an undesirable allele in their population by 50% on each generation. Once this elite breeding herd is free, the original selection has paid off, when semen from these elite stud boars is used in multiplier and commercial herds. Associated with gender selection, freezing of marker-assisted selected stud sire herds appears extremely synergistic.

Cryopreservation of semen is, however, suboptimal since variation between species, individuals, and even between sperm subpopulations is so clearly marked. Selection pressure put on freezability is not always possible. It has worked for dairy bulls (one basic freezing method is used to discriminate among young sires) but will probably not be used with the same impetus when fewer bulls are to be recruited by genome selection of calves, as it is applied at present. We would therefore see situations similar to other species, where semen cryopreservation methods are "adapted" for a particular sire in order to "save" the genetic value it represents. Preselection of the most robust spermatozoa is considered for instance to increase the use of otherwise "weak" sires (as in equine), but will not be effective in other species unless related to sex-sorting, of functional genomic selection. Despite suboptimality,



conventional slow equilibrium freezing methods are thus to continue being the major option. Alternative methods such as vitrification (which avoid the risk of ice damage and solute toxicity) are less likely to be applied simply because of the size of the sperm sample that needs to be frozen, unless a breakthrough is reached. The same applies for freeze-desiccation since the number of sperm needed for the only commercially competitive method of AI is too large. On the other hand, both vitrification and sperm-drying of small sperm suspension volumes are realistic options for IVF and production of embryos *in vitro* for ET, in any livestock species and most realistic in equine, where ICSI is routinely practiced.

Diagnostic tools for semen assessment are going to continue developing, involving both spermatozoa and the surrounding SP. Whether this will require use for sophisticated instrumentation (as flow cytometers still are) or if the level of simplification in this sector with advance more rapidly that what has been the case leading to more accessible bench- or field instruments is yet to be awaited. Most likely we shall see cheaper, operator-friendly instruments in a few years. Ultimately, development in terms of semen biotechnology will depend on the costs it implies for the designers and the enterprises. After all, semen is still the “cheapest” component of livestock breeding.

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## Molecular and endocrine determinants of oocyte competence

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### Abstract

Mammalian female gametes are stored for many years in the ovaries in an inactive state until stimulated to grow. Activation of the resting follicle is initiated by the oocyte and requires a communication mechanism between the oocyte and the surrounding granulosa cells. Key molecular and morphological events that occur during oocyte and follicle growth and maturation include the establishment of a bi-directional communication system, granulosa cell proliferation and antrum formation, nucleolus activation and initiation of oocyte transcription, establishment of the maternal imprints and completion of the meiotic maturation. Successful accomplishment of these events furnishes the oocyte with the competency to support fertilization and to sustain early embryo development and is reflected in the molecular signatures of the oocyte and the follicular granulosa cells. The current review will consider these events and highlight the molecular and endocrine factors associated with them.

**Keywords:** egg, follicle, gene expression, progesterone.

### Introduction

Mammalian female gametes are stored in the ovaries as inactive oocytes, arrested at the diplotene stage of meiosis and surrounded by a single layer of flattened granulosa cells until they are stimulated to grow (Erickson, 1966; Fair *et al.*, 1997a). Oocyte storage can be extremely prolonged, spanning the fetal, prepubertal and reproductive lifetime of the animal. The classical studies of Lussier *et al.* (1987), indicate that in cattle, progression from the primary follicle to the tertiary follicle stage takes approximately 60 days and about 2 estrous cycles are required for a follicle to grow from antrum formation to preovulatory size. Oocyte meiotic arrest is maintained throughout follicle activation and subsequent initiation of oocyte growth and folliculogenesis. During the periovulatory period, the oocyte, completes maturation and resumes meiosis in response to luteinizing hormone (LH). Despite a relatively short duration of 24 h in cattle, the changes in the endocrine milieu and subsequent dramatic changes in the morphological and biochemical profiles of both the follicle and the oocyte which characterize the periovulatory period, profoundly affect the oocyte's competence to support fertilization and embryogenesis

and the follicle's subsequent performance as the corpus luteum. The current review will highlight the key determinants of mammalian postnatal oocyte growth and development, specifically focusing on the events associated with the completion of the growth phase, maturation, and ovulation in cattle.

### Activation of follicle growth

Conventional knockout or oocyte-specific deletion studies have facilitated the identification of several factors such as *Sohlh2*, anti Müllerian hormone (AMH) and *Pten*, as repressors of follicle activation, (Sun *et al.*, 2008 for review). Early studies, suggested that follicle and oocyte growth was in response to signals from the granulosa cells, however, the identification of the oocyte-secreted transforming growth factor beta (TGF $\beta$ ) superfamily member, growth differentiation factor 9 (GDF9), led to the revelation of the oocyte as the driving force in development beyond the primary follicle stage (Eppig, 1991; Dong *et al.*, 1996). Subsequent studies identified another factor, the oocyte-secreted and structurally related family member, BMP15 (GDF9B; Dube *et al.*, 1998). Sequential profiling of the murine oocyte transcriptome according to stage of folliculogenesis, has identified that the transition from primordial to primary follicle is associated with the greatest change in the oocyte transcriptome profile. In agreement with the pioneering studies mentioned above, the key factors that were upregulated in oocytes following transition to the primary follicle stage were members of the TGF- $\beta$  superfamily, including *Bmp15*, *Gdf9*, *Bmp5*, *Bmp6*, *Tgfb2*, and *Tgfb3*, as well as other growth factors such as *Kitld*, *bFgf*, and *Lif* (Pan *et al.*, 2005). Bi-directional communication between the oocyte and the granulosa cells is critical to follicle activation; in cattle this appears to be facilitated in the primordial and primary follicles through receptor-mediated endocytosis, as gap junctions are absent and the oolemma is characterized by the presence of numerous coated pits and coated vesicles in the cortical ooplasm (Fair *et al.*, 1997a).

### Preantral follicle development

In cattle, establishment of a direct communication system commences during the progression from the primary to the secondary follicle stage, when gap junctions are formed between the



surface membranes of the oocyte and the surrounding granulosa cells (Fair *et al.*, 1997a). Additional key morphological changes occurring at the secondary follicle stage include deposition of zona pellucida material around the oocyte, synthesis of cortical granules within the oocyte cytoplasm, nucleolus reorganisation and activation, and the first detection of RNA synthesis (Fair *et al.*, 1997a, b). It is at this point in cattle and sheep, that FSHr mRNA expression was first detected (Tisdall *et al.*, 1995; Xu *et al.*, 1995; Bao and Garverick, 1998), implying the establishment of responsiveness to gonadotropins. The transition from the secondary follicle to the tertiary follicle stage is characterized by the continued proliferation and differentiation of the somatic cells surrounding the oocyte to form the theca interna and externa and the basal lamina. At the same time oocyte volume quadruples accompanied by proliferation of the oocyte organelles and driven by intensive mRNA and rRNA transcription (Fair *et al.*, 1995, 1996, 1997a). These events appear to be driven by TGF- $\beta$  superfamily members; granulosa cell-derived activin, BMP-2, -5 and -6, theca cell-derived BMP-2, -4 and -7, and oocyte-derived BMP-6 promote granulosa cell proliferation, follicle survival and prevention of premature luteinization and/or atresia, respectively (Knight and Glistler, 2006). GDF9 deletion studies in mice showed that oocyte-derived GDF9 is required for the formation of transzonal projections (TZPs; Dong *et al.*, 1996). TZPs form a core structural component of an elaborate bi-directional intercellular communication system between the oocyte and surrounding somatic cells that develops during follicle development and functions to meet the metabolic requirements of the growing oocyte (Eppig, 1991; Carabatsos *et al.*, 1998; Li and Albertini, 2013). GDF9 and GDF9b have been identified as major players in cumulus cell metabolism, particularly glycolysis and cholesterol biosynthesis (Su *et al.*, 2008). Other key factors expressed in the granulosa cells at this time include Insulin-like growth factor (IGF), IGFBP2, IGFBP3, and Type 1 IGF receptor (Armstrong *et al.*, 2003), LHr (Xu *et al.*, 1995), steroidogenic enzymes P450scc, P450c17, and 3 $\beta$ -HSD (Bao and Garverick, 1998). Follicles become increasingly FSH responsive and their growth and continued survival becomes increasingly gonadotropin dependent.

#### *Antral follicle growth - oocyte transcription, maintenance of meiotic arrest and establishment of maternal imprints*

In cattle, antrum formation occurs when follicles reach a diameter of 130-200  $\mu$ m and is completely gonadotropin -dependent. Once formed, the size of the antrum increases rapidly and the granulosa cells proliferate and differentiate into mural granulosa cells and cumulus cells which surround the oocyte (Lussier *et al.*, 1987; Fair *et al.*, 1997a). FSH granulosa

cell differentiation appears to be indirectly induced by FSH, through the activation of the PI3-K/AKT pathway (Fan *et al.*, 2008).

#### *Oocyte RNA transcription:*

Early tertiary follicle stage oocytes are transcriptionally active and display at least one active nucleolus. Transcriptional activity continues as oocyte and follicle growth proceeds, the successful accumulation of messenger RNAs (mRNAs), ribosomes and polypeptides by the oocyte ultimately affects the developmental potential of the resulting ovulated egg (see review Fair, 2003). Oocyte transcription declines when oocyte diameter reaches 110  $\mu$ m, the decline in transcription is associated with the completion of the oocyte growth phase, as oocyte diameter subsequently plateaus at approximately 120-130  $\mu$ m, corresponding to a follicle diameter of 3 mm (Fair *et al.*, 1995). Oocyte diameter will be maintained throughout maturation, fertilization, and development up to the early blastocyst stage, in contrast, the follicle can grow up to 15-20 mm in diameter before ovulation. Transcriptomic profiling of fully grown oocytes from antral follicles with differing developmental potential has identified pathways associated with RNA processing and the control of chromosome segregation to be associated with oocyte competence (Labrecque *et al.*, 2013). While the analysis of the oocyte transcriptome provides valuable information, the oocyte must be sacrificed in order to carry out the analysis. Therefore, the identification of non-invasive indicators of oocyte competence would aid in the selection of optimal oocytes for assisted reproduction therapies. The analysis of follicular parameters revealed that intrafollicular steroid concentrations were not predictive of oocyte developmental potential; however, the metabolomic profile of the follicular fluid, particularly the amino acid profile was highly predictive (Matoba *et al.*, 2013).. Greater mRNA abundance of LHCGR in granulosa cells, ESR1 and VCAN in thecal cells and TNFAIP6 in cumulus cells was also associated with highly competent oocytes from antral follicles (Matoba *et al.*, 2013).

#### *Maintenance of meiotic arrest*

The interaction of mural granulosa cells, cumulus cells, and oocytes is essential for maintaining oocyte meiotic arrest in the fully grown oocyte, prior to the LH surge. Inhibition of oocyte cAMP-phosphodiesterase (PDE3A) activity is essential for sustaining elevated oocyte cAMP concentrations, which is crucial for maintaining meiotic arrest. Cyclic GMP diffuses into the oocyte from companion cumulus cells via gap junctions and inhibits oocyte PDE3A activity and cAMP hydrolysis, thus maintaining meiotic arrest (Norris *et al.*, 2009; Vaccari *et al.*, 2009). Recently,



work in mice has identified Natriuretic peptide precursor type C (NPPC) as a key factor in this process. NPPC is produced by follicular mural granulosa cells, it stimulates the generation of cGMP by the cumulus NPPC receptor, NPR2, which is required to inhibit oocyte PDE3A activity and thereby maintain meiotic arrest (Zhang *et al.*, 2010).

#### *Establishment of maternal imprints*

According to data from mice (Lucifero *et al.*, 2002; Hiura *et al.*, 2006), human (Geuns *et al.*, 2003) and cattle (O'Doherty *et al.*, 2012), DNA methylation of the maternal imprints occurs during the oocyte growth phase. The correct establishment and maintenance of methylation patterns at imprinted genes has been associated with placental function and regulation of embryonic/fetal development whereas abnormal reprogramming of maternal and/or paternal imprinted loci has been associated with reduced developmental potential. In mice, the onset of methylation coincides with the transition from primary to secondary follicles or as oocytes attain a diameter of >50  $\mu\text{m}$ . In cattle, methylation onset may occur later, as maternal imprints were incompletely methylated in oocytes <100  $\mu\text{m}$ . DNA methylation appears to be a progressive process, such that partially methylated intermediates can be detected in populations of oocytes (Tomizawa *et al.*, 2011). In addition, the timing of DNA methylation appears to be imprint specific (O'Doherty *et al.*, 2012). In mice, CpG methylation is completed by the time oocytes reach >70  $\mu\text{m}$  (Smallwood *et al.*, 2011), and in cattle completion appears to occur in parallel with cessation of transcription. DNA methylation patterns are established and maintained by a family of enzymes, the DNA methyltransferases (DNMTs). They consist of five members: DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L. DNMT1 is regarded to be the maintenance methyltransferase (Yoder *et al.*, 1997; Ooi *et al.*, 2009), whereas the DNMT3 enzymes are required for establishing methylation during gametogenesis and early embryonic development (Okano *et al.*, 1999). DNMT3L is highly expressed in bovine oocytes during the critical period of DNA methylation imprint acquisition, suggesting that it plays a role similar to that described in mouse (Bourc'his *et al.*, 2001; Kaneda *et al.*, 2004; O'Doherty *et al.*, 2012) in the establishment of maternal imprints.

#### *Preovulatory follicle - Cumulus cell expansion, oocyte maturation, endocrine factors:*

In cattle the LH surge induces luteinization, cumulus cell-oocyte complex (COC) expansion, final oocyte maturation, ovulation and a change in the follicular endocrine environment from E2 dominance to P4 dominance in the follicular fluid. Oocyte maturation is characterized by the breakdown of the oocyte nuclear membrane, causing the release of the nuclear contents

into the cytoplasm, subsequent chromosome condensation, extrusion of the first polar body, and meiotic arrest at metaphase II (Fair, 2003 for review). Because oocytes express few or no LH receptors, it would appear that LH stimulates the (LH receptor-positive) mural granulosa cells to produce epidermal growth factor-like factors Areg, Ereg, and Btc that act on the LH receptor-negative cumulus cells in concert with GDF9/BMP15 to propagate LH signaling throughout the preovulatory follicle (Park *et al.*, 2004). The direct effects of the GDF9/BMP15 and indirect effects of the LH signaling pathway through the mural granulosa promote cumulus expansion. Granulosa and cumulus cell depletion of extracellular signal-regulated kinase (ERK) 1 and 2 (also known as mitogen-activated protein kinases 1 and 3) indicate their central role in cumulus cell expansion, oocyte maturation and ovulation in response to LH (Fan *et al.*, 2009). In the same study, the transcription factor CCAAT box enhancer binding protein-beta (C/EBP- $\beta$ ) was identified as a downstream effector of ERK1/2 in granulosa cells during ovulation and luteinization; target genes include Cyp19a1 and Sult1e1, which regulate estradiol biosynthesis and activity; Star and Cyp11a1, which are associated with granulosa cell luteinization; the EGF-like factors Areg, Ereg and Btc and the cumulus expansion factors Ptgs2, Tnfrsf6, Has2, Ptx3 and Pgr. Cumulus expansion aids in the expulsion of the oocyte from the ruptured follicle and also facilitates chemoattraction of sperm towards the oocyte (Chang and Suarez, 2010).

Meiotic maturation is controlled by three key biochemical pathways: (1) maturation-promoting factor (MPF) which activates meiotic resumption resulting in germinal vesicle breakdown (GVBD); (2) anaphase promoting complex (APC) which regulates progression from GVBD through the subsequent stages of meiosis; and (3) cytostatic factor (CSF) which maintains meiotic arrest at MII. Regulation of MPF is mostly via the activity of several kinases and phosphates, whereas APC and CSF are regulated mainly by translational regulation through sequential waves of polyadenylation and deadenylation (Belloc *et al.*, 2008). A recent meta-analysis of previously published microarray data on various models of metaphase II -stage oocyte quality led to the identification of 56 candidate genes associated with oocyte quality across several species (O'Shea *et al.*, 2012). One of the most striking aspects of the analysis was the differential expression of genes linked to mRNA and protein synthesis between models, underlining the importance of de novo protein synthesis and its regulation for successful oocyte maturation and subsequent development. Several genes that are known to be involved in oocyte maturation and/or embryonic developmental competence such as GDF9, EMI1, and PTPN1 were identified. Nuclear maturation alone is not sufficient to produce a high quality oocyte; bioinformatic analysis of preferentially populated



pathways identified Wnt signaling and the antiapoptotic PI3K/Akt pathway as being key to the maturation of a high quality oocyte. Regulation of the canonical Wnt/beta-catenin pathway interlinks with APCregulation and metaphase II arrest of an oocyte.

Factors produced by the cumulus cells and/or components of the follicular fluid such as progesterone (P4), estrogen (E2), insulin, and IGF play an important role in oocyte and follicle development. For example, during development to the preovulatory stage, intrafollicular E2 increases granulosa cell mitosis, promotes gap junction formation among granulosa cells, increases the stimulatory action of FSH on aromatase activity and regulates the expression of several steroidogenic enzymes and induces FSH and LH receptor expression in granulosa cells (Geary *et al.*, 2012 for review). High concentrations of E2 within the follicular microenvironment may impact bovine oocyte maturation and competence. Data from IVP studies indicate that preovulatory follicular fluid E2 concentrations are associated with higher blastocyst development rates following in vitro maturation, fertilization, and culture (Mermillod *et al.*, 1999; Van De Leemput *et al.*, 1999). The switch from E2 dominance to P4 dominance in mammalian preovulatory follicular fluid in the period between the LH surge and ovulation (Dieleman *et al.*, 1983) and COC cumulus cell P4 synthesis during IVM (Aparicio *et al.*, 2011; Salhab *et al.*, 2011) coincident with resumption of meiosis and maturation of the oocyte, implies a role for P4 in oocyte maturation. Inhibitory experiments have confirmed the functional relevance of P4 and P4 receptor signaling during oocyte maturation to oocyte acquisition of developmental competence. The membrane bound receptors PGRMC1 and mPR $\alpha$  appear to be involved in oocyte meiotic maturation and first mitosis, respectively, as intracytoplasmic injection of oocytes with an antibody against PGRMC1 affected chromosome segregation during oocyte meiotic maturation (Luciano *et al.*, 2010) and the addition of an mPR $\alpha$  specific antibody during IVM reduced the percentage of oocytes progressing through the early cleavage stages (Aparicio *et al.*, 2011). Furthermore, nuclear progesterone receptor (PRG) signaling during oocyte maturation appears to be important for oocyte developmental competence as blastocyst development rates were dramatically reduced when either cumulus cell P4 synthesis was inhibited using Trilostane, or PRG signaling was blocked using RU 486 during IVM (Aparicio *et al.*, 2011). Further investigations are required to determine the mechanism(s) by which P4 'promotes' acquisition of developmental competence. Initial investigations by our group suggest P4 acts to protect the oocyte from apoptosis. Indeed several studies have indicated a pro-survival or anti-apoptotic role for P4 in female reproductive tissues; for example, P4 has been shown to inhibit apoptosis in the uterus (Wang *et al.*, 2003), the corpus luteum (Okuda *et al.*,

2004; Liszewska *et al.*, 2005), and the ovarian follicle (Besnard *et al.*, 2001). The main anti-apoptotic action of P4 was demonstrated to be mediated via the classical PGR in periovulatory rat granulosa cells as treatment of these cells with the PGR antagonist Org 31710 induced increased caspase 9- and caspase 3/7 activity (Friberg *et al.*, 2009, 2010). At the level of the COC, in vitro maturation in media that promoted cumulus cell P4 synthesis resulted in lower rates of cumulus cells apoptosis and higher oocyte competence (Salhab *et al.*, 2011). Additional studies have described a role for PGRMC1 in the mediation of the anti-apoptotic effects of P4 and sterol metabolism (Gilchrist *et al.*, 2004; Hussein *et al.*, 2005) implying that anti-apoptotic effects of P4 are not confined to one pathway but are due to the regulation of several key pathways and processes occurring during oocyte maturation.

### Conclusion

The development of the oocyte from the resting primordial stage up to the fully competent ovulated egg involves the progression through a number of developmental checkpoints which are under the control of several key factors. These factors include: members of the TGF- $\beta$  superfamily, particularly GDF9 and GDF9B, which play pivotal roles in the establishment of bi-directional communication between the oocyte and the granulosa cells, follicle activation, and initiation of transcription in the oocyte; NPPC and NPR2 which are critical to the maintenance of oocyte meiotic arrest during the oocyte phase; DNA methyltransferase enzymes which establish and maintain the maternal imprints in the oocytes, a key checkpoint in the completion of the oocyte growth phase; and finally P4 which promotes meiotic and cytoplasmic maturation and acts to protect the integrity of the oocyte genome for the next generation.

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## Sanitary requirements for bovine gametes and embryos in international trade

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### Abstract

Development of artificial insemination (AI) together with embryo cryopreservation has led to international trade of cattle germplasm for more than 60 years. Although experimental data show that many animal pathogens can be associated with semen and embryos, risk of disease transmission can be substantially reduced or eliminated by applying sanitary protocols recommended by the International Embryo Transfer Society (IETS) and the World Organization of Animal Health (OIE). The basic principle to ensure such a high level of biosecurity for semen relies on the concept of pathogen-free semen collection center. In the case of embryos, practical guidelines have been published in the manual of IETS in order to provide risk management procedures ensuring the safety of herds using embryo transfer, and embryo washing procedures which are the most effective means of reducing the number of microorganisms associated with germplasm. Finally, the high degree of biosecurity measures under official approval ensures that the professionalism of embryo transfer (ET) teams and good AI industry practices together with the low risk of disease transmission using gametes and embryo based biotechnologies, encourages germplasm movement around the world.

**Keywords:** biosecurity, embryo, sanitary protocols, semen.

### Introduction

Development of artificial insemination (AI) along with embryo cryopreservation has led to large-scale exchange of cattle germplasm over the past 60 years, thus taking advantage of financial, sanitary, and animal welfare aspects compared to movement of live animals. A recent review estimated that approximately 50 million doses of bovine semen with a value of US\$250 million, and approximately 80,000 bovine embryos with a value of about US\$15 million, are traded internationally on an annual basis (Thibier and Wrathall, 2012). Although these data are approximations, they do indicate that there has been a substantial increase in trading bovine semen and embryos over the last decade. The major semen-exporting countries are the United States with a value of approximately US\$81.2 million), Canada with

a value of approximately US\$78.3 million and the European Union with a value of approximately US\$76 million. Indeed, there are worldwide opportunities to develop international trade of livestock germplasm. As an example, the Foreign Agricultural Service (FAS) of the United States Department of Agriculture (USDA) worked with its Animal and Plant Health Inspection Service (APHIS) to negotiate export health certificates, allowing for the export of live cattle, semen, embryos, horses, and swine to Russia for the first time in 2008. This market to Russia was valued at nearly US\$12 million in 2010. From January to May 2011, trade increased nearly fivefold compared to the same period in 2010 (USDA Foreign Agricultural Service, 2008; USDA Blog, 2011).

As experimental data show that many animal pathogens can be associated with semen and embryos (Bielanski, 2006; Van Soom *et al.*, 2010) the basic principle to ensure a high level of biosecurity for semen relies on the concept of pathogen-free semen collection center (Thibier and Guérin, 2000). In case of embryos, embryo washing procedures as described in the IETS Manual are the most effective in reducing the number of microorganisms associated with germplasm. These disease control measures have been identified and assessed by the IETS Health and Safety Advisory Committee, the expert body that advises the OIE on matters related to sanitary procedures in embryo transfer (Thibier, 2011).

This review will focus on the sanitary and hygiene requirements for semen and embryos in international trade. Variations between regulatory and sanitary requirements will be described as well as possible consequences on safety of semen and embryos.

### Sanitary requirements for semen collection and international trade

As a general statement, the goal is to limit the risk of transmission of any animal disease through artificial insemination. Semen must be collected and processed at approved and supervised semen collection centers, obtained from animals whose health status ensures there is no risk of spread of any animal disease through artificial insemination, and collected, processed, stored, and transported in accordance with regulations which preserve its health status.

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*General requirements: industry self-regulation, approval, and official supervision*

In the world, general requirements are described in the OIE Terrestrial code for semen collection, processing, and storage centers (SCC = Semen Collection Center; SSC = Semen Storage Center) in two dedicated chapters (OIE, 2012; Chapters 4.5 and 4.6).

Some differences exist among the requirements of the OIE, Certified Semen Services (CSS) or European Union (EU) as for example, frequency of audits, scheduled once or twice a year (Table 1), and the approval procedure or the supervision, which may be assumed directly by official veterinarians or partly delegated to self-control institutes.

Table 1. General requirements for veterinary and official supervision.

References	Requirements
OIE requirements	AI center officially approved by the Veterinary Authority.
OIE, 2012 (Chapter 4.5)	<ul style="list-style-type: none"> <li>under the supervision and control of the Veterinary Services which will be responsible for regular audits, <u>at an interval of no more than 12 months</u>, of protocols, procedures and records on the health and welfare of the animals in the center and on the hygienic production, storage and dispatch of semen.</li> </ul>
CSS requirements	AI center, under the direct supervision and control of a center veterinarian.
Certified Semen Services - CSS, 2011a (Agreement)	<ul style="list-style-type: none"> <li>AI Center (Stud) code number assigned by the National Association of Animal Breeders;</li> <li><u>annual Semen Identification Audit</u> by a representative of CSS and possibly an accompanying representative from USDA, with access to all phases of semen production and related identification functions;</li> </ul>
Certified Semen Services - CSS, 2011b (AI center animal)	<ul style="list-style-type: none"> <li>additional USDA-APHIS Certificate for the approval in accordance with Council Directive 88/407/EEC of a semen collection center.</li> </ul>
EU requirements	All approved semen collection centers (SCC) registered, with a veterinary registration number.
European Union, 1988 (Council Directive 88/407/EEC)	<ul style="list-style-type: none"> <li>list of SCCs and their veterinary registration numbers sent to the Commission (Decision 2007/846/EC);</li> <li>notification of any withdrawal of approval;</li> <li>inspections by an official veterinarian, <u>at least twice a year</u>, at which time standing checks on the conditions of approval and supervision shall be carried out.</li> </ul>
	SCC under the <u>permanent supervision</u> of a center veterinarian.

Within the EU, Article 3(a) of Council Directive 88/407/EEC (European Union - EU, 1988) requires SCCs and SSCs to be approved, when they collect or store semen which may enter intra-community trade. The same applies to embryo collection teams or embryo production teams, according to Article 3(C) of Directive 89/556/EEC. Article 5 in each Directive requires that each center, or team, is given a veterinary registration number, and that the approval conditions are under official supervision. An updated list of approved teams and centers must be sent to the other Member States (as described in Decision 2007/846/EC).

The Food and Veterinary Office (FVO) works to assure effective control systems and to evaluate compliance with standards within the EU, and in third countries in relation to their exports to the EU. The FVO does this mainly by carrying out inspections in Member States and in third countries exporting to the EU. Each year the FVO develops an inspection program, identifying priority areas and countries for inspection. In order to ensure that the program remains

up-to-date and relevant, it is reviewed mid-year. These programs are published on a website ([http://ec.europa.eu/food/fvo/index\\_en.cfm](http://ec.europa.eu/food/fvo/index_en.cfm)). The findings of each inspection carried out under the program are set out in an inspection report, together with conclusions and recommendations. The competent authority of the country visited is given the opportunity to comment on the reports at draft stage.

Certified Semen Services (CSS), Inc., is a wholly owned subsidiary of the National Association of Animal Breeders (NAAB) in the USA. The CSS program has enabled the national animal breeding industry to regulate itself without the direct government involvement. CSS is organized so that any AI business engaged in collection and processing of livestock semen is eligible to participate in and benefit from its services program upon entering an agreement for services. The CSS Service Director annually makes at least one unannounced audit visit to the offices and semen production facilities of each participating AI business. During the audit visit, procedures and records related to



semen identification and sire health are reviewed. A complete report of this review or audit is provided to the president and manager of the AI business audited. The audit report is confidential between CSS and the participating organization (CSS, 2011a).

#### *Facilities and isolation requirements*

Facilities must facilitate the separation of resident animals (used for semen collection) from sick

animals and farm livestock on adjacent land or buildings, as described in OIE Terrestrial Animal Health code and in EU requirements (Table 2). In the CSS (2011a), an enclosed laboratory used for semen processing, partitioned from bull housing and semen collection areas is described. All facilities and their management procedures should provide safety for both bulls and handlers. Facilities should be designed and lighted to permit easy visual observation of the population, with fences designed to effectively and safely contain bulls.

Table 2. Facility requirements in semen collection, processing and storage centers (SCC = Semen Collection Center; SSC = Semen Storage Center; CSS = Certified Semen Services).

References	Requirements
OIE requirements	<ul style="list-style-type: none"> <li>• AI center: animal accommodation areas (species specific);</li> <li>• isolation facility for sick animals;</li> </ul>
OIE, 2012 (Terrestrial Animal Health Code; Chapter 4.5)	<ul style="list-style-type: none"> <li>• semen collection room;</li> <li>• separate and distinct areas for accommodating resident animals, for semen collection, for feed storage, for manure storage, and for isolation of animals suspected of being infected;</li> <li>• a semen laboratory and semen storage areas;</li> <li>• administration offices;</li> <li>• a pre-entry isolation facility (not compulsory in case of horses).</li> <li>• only animals associated with semen production permitted to enter the center (see Table 4);</li> <li>• other species of livestock exceptionally resident on the center, provided that they are kept physically apart from these animals;</li> <li>• donors and teasers in the center adequately isolated from farm livestock on adjacent land or buildings e.g., by natural or artificial means.</li> </ul>
CSS, 2011a (requirements, in CSS agreement)	<p>Fully enclosed laboratory used for semen processing, partitioned from bull housing and semen collection areas;</p> <ul style="list-style-type: none"> <li>• structured to provide for hygienic handling and storage of semen.</li> </ul>
EU requirements, EU, 1988 (Council Directive 88/407/EEC)	<ul style="list-style-type: none"> <li>• animal housing including isolation facilities;</li> <li>• semen collection facilities including a separate room for the cleaning and disinfection or sterilization of equipment;</li> <li>• a semen processing room;</li> <li>• a semen storage room;</li> <li>• isolation accommodation, no direct communication with the normal animal accommodation;</li> </ul> <p>The SCC must be so supervised that only semen collected at an approved center is processed and stored in approved centers, without coming into contact with any other consignment of semen.</p>

As a general rule, the SCC must be so constructed so that the animal housing and the semen collecting, processing, and storage facilities can be readily cleaned and disinfected. Facilities that enable a “forward process” allow for separation between animal linked personal and semen streams leading to more control of the risk of contamination (Fig. 1).

#### *Records related to bull and semen traceability*

Requirements regarding bull and semen identification ensure records of health tests and the

consequent biosecurity (Table 3). Similar requirements are described in the CSS agreement, as well as in the EU directive regarding bull health records and semen identification.

Bull and semen movement have increased as a result of increased trade and the use of sexed semen. As a consequence, traceability between semen collection and processing centers and movement between barns become more critical. In some countries, e.g., France, additional requirements concerning traceability are described in the Ministerial Order (France, 2008). Moreover, the French National Database for Health



Control of breeding animals is an interesting tool, enabling the storage of data as individual characteristics (e.g., breed, date of birth and the name, identification, pre-entry station, SCC, and movement of livestock), health data (complete records of health checks), and

movement of semen and animals. A website provides a simple way for competent authorities and breeding companies to access the complete records of each bull ([www.lncr.org](http://www.lncr.org)). Moreover, this interactive system is intended to limit the amount of paper certificates.

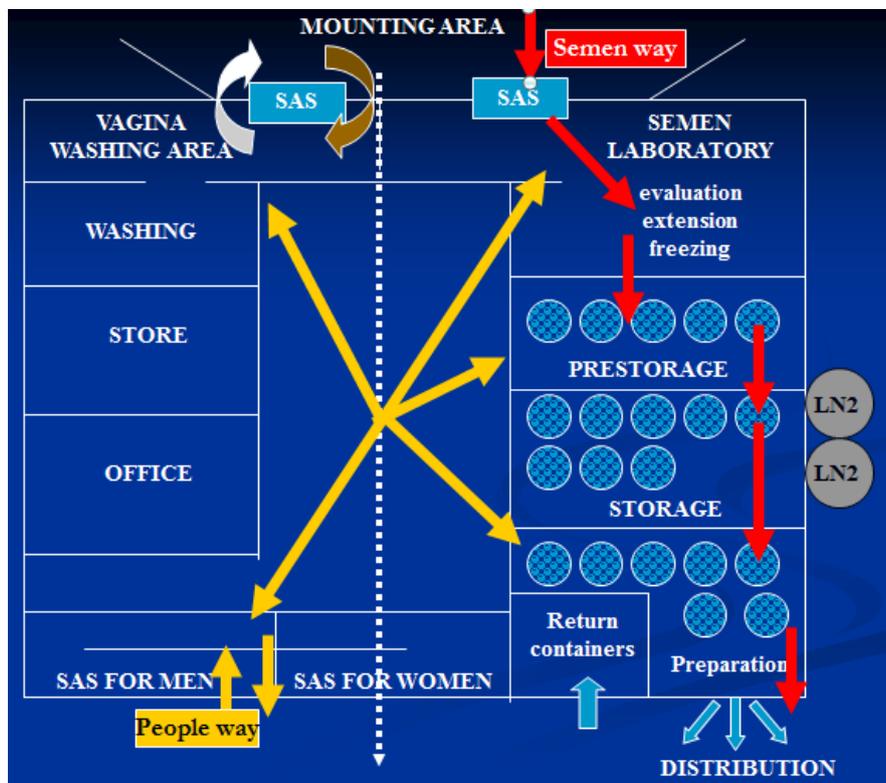


Figure 1. Forward process and separation between people and semen streams in a semen collection center. Adapted from Thibier and Guérin (2000).

Table 3. Data records enabling bull and semen traceability.

Source	Requirements
CSS requirements	<ul style="list-style-type: none"> <li>records for a period of 6 years showing all sire purchases and leases, semen collections and shipments;</li> </ul>
CSS, 2011a (agreement)	<ul style="list-style-type: none"> <li>records of original sale of semen, including sale in conjunction with insemination.</li> <li>use of prenumbered CSS Approved Sales Invoices;</li> <li>records of health tests completed on each bull for a period of 6 years from the date of such tests;</li> <li>identification of semen: (1) Registration name and number of the bull, (2) Collection code, (3) A.I. Center (Stud) code number assigned by the National Association of Animal Breeders, (4) Breed Code, (5) Bull's number assigned by the A.I. Business.</li> </ul>
EU requirements	<ul style="list-style-type: none"> <li>record of all bovine animals at the center, giving details of the breed, date of birth and identification of each of the animals;</li> </ul>
EU, 1988 (Council Directive 88/407/EEC)	<ul style="list-style-type: none"> <li>record of all checks for diseases and all vaccinations carried out, giving also information from the disease/health file of each animal;</li> <li>identification of semen: (1) date of collection of the semen, (2) breed and identification of the donor animal, (3) name of the center, (4) characteristics and form in accordance with Article 19.</li> </ul>



### *Technical staff and animal management*

As mentioned in the OIE Animal Health Code, the laboratory personnel should be technically competent and observe high standards of personal hygiene to preclude the introduction of pathogenic organisms during semen evaluation, processing, and storage. In CSS (2011b; AI center animal management guidelines), complete recommendations for animal management, sire and hygiene procedures, feeding and housing conditions and veterinary and professional care are described.

### *Specific sanitary requirements for bovine semen*

In general terms, microorganisms can be present in the semen of an infected male or can gain entry to semen during collection, processing, or storage. In order to maintain a controlled status of semen batches, tests must be applied on semen and genital tract, semen production, and sanitary control of the bulls present in the center.

For given pathogenic agents, semen can be certified as free if the donor bull originated from a free herd, the dam of the bull is free, the donor bull is free, the donor bull is introduced to a center where all other bulls are free, and all the bulls present in the center are submitted to regular tests regarding this agent. For each agent, specific OIE requirements aim to maintain the health of animals on an artificial insemination center at a level which permits the international distribution of semen with a negligible risk of infecting other animals or humans with pathogens transmissible by semen, as described in the Table 4.

### **Sanitary requirements for embryos used in international trade**

Although transfer of bovine embryos is much less likely to result in disease transmission than transport of live animals (Thibier and Wrathall, 2012), the sanitary risk associated with bovine embryo transfer remains the subject of scientific investigations (Van Soom *et al.*, 2008) and adaptations of national and international legislations (OIE, 2012; Chapters 4.7 and 4.8).

### *Physiological background*

Interaction of oocytes and embryos with pathogenic agents has been extensively reviewed by Bielanski (2006). Oocytes and early embryo stages up to approximately day 8 after fertilization, are surrounded by an acellular glycoprotein shell with a sponge-like surface, the zona pellucida (ZP). Visualized by scanning electron microscopy, the ZP is composed of a fibrous network with numerous pores. The pores are larger at the outer surface (outer diameter of embryos range from 155 to 223  $\mu\text{m}$  for different embryonic developmental stages) but decrease in size centripetally in both animals and human embryos (Bielanski, 2006). Such anatomical structures of the ZP allows for adhesion of pathogens, but prevents them from fully penetrating the ZP.

Since the ZP is acellular in character, viruses are

not able to replicate there and they must cross the ZP and the cell plasma membrane to infect an embryo. In general, ova or embryos can become contaminated at different stages: before the ZP is formed (under physiological conditions, the ZP is formed in the ovarian preantral secondary follicles), later by agents present in the follicular fluid, by pathogen-contaminated semen during fertilization or during passage through the oviduct and the uterus, even if integrity of the ZP prevents contamination of embryonic cells for most pathogens (Bielanski, 2006; Van Soom *et al.*, 2008). With the application of *in vitro* fertilization techniques, immature oocytes surrounded by a multilayer of compacted granulosa cells (cumulus-oocyte complexes) are collected from ovarian follicles using Ovum Pick Up (OPU) or from slaughterhouse derived ovaries and placed in the maturation medium. During this period, the granulosa cells become expanded and their connections with the ZP loosen. Later, the granulosa cells are mechanically removed, deleting the connection between these cells and the ZP. Specific risks are linked to artificial culture conditions, rather than the utero-tubal environment which occurs with *in vivo* fertilization (Bielanski, 2006).

### *Requirements applied to in vivo derived embryos*

#### *Assessment of disease transmission via in vivo derived embryos*

Experience and experimental evidence has indicated a low potential for transmission of infectious pathogens via *in vivo*-derived (IVD) embryos (Givens *et al.*, 2007; Thibier 2011). Pathogens may be shed into the genital tract and contaminate the surface of embryos, if those pathogens are present at the time of collection or between fertilization and collection. Scientific reflections on the sanitary risks associated with ET have focused on the probability that embryos can be contaminated either via the oocyte, the semen, or adhesion to the zona pellucida. There have been many investigations to evaluate interactions between pathogens and embryos, using different *in vivo* or *in vitro* infection approaches (Table 5).

*In vivo* approaches are the most suitable to evaluate the likelihood of transmission through embryo transfer, however such experiments require expensive protocols in order to infect donor animals and perform subsequent transfer to recipients. As an example, facilities with a controlled environment and that are insect-proof are required to investigate vector transmitted diseases. For diseases with a long incubation period (e.g., prions), experiments may last several years. Complementary *in vitro* approaches can be used to gain knowledge with more accessible costs (Table 5). Indeed, *in vitro* experiments are ultimately more conservative and are not as likely to be influenced by factors like minimum infective dose, innate immunity etc. Realistically, if an *in vitro* experiment does not reveal the presence of infectious agents, the likelihood of an *in vivo* experiment showing its presence is very unlikely.



Table 4. Specific OIE requirements for international distribution of semen with a negligible risk of infecting other animals or humans with pathogens transmissible by semen (OIE, 2012).

Diseases	Semen
Bovine Brucellosis (BB)*	<ul style="list-style-type: none"> <li>• semen from an AI center: testing program with the buffered Brucella antigen (rose Bengal test; RBT) and complement fixation tests (CFT);</li> <li>• semen not issued from an AI center: country or zone free from BB; or herd officially free from BB, no clinical sign of BB on the day of collection of the semen and animals subjected to a RBT with negative results over the 30 days prior to collection; or herd free from BB, no clinical sign of BB on the day of collection and animals subjected to RBT and CFT with negative results during the 30 days prior to collection.</li> </ul>
Bovine Genital Campylobacteriosis (BGC)	<ul style="list-style-type: none"> <li>• donor animals have never been used for natural service; or have only mated virgin heifers; or kept in an establishment or AI center where no case of BGC has been reported; culture of semen and preputial specimens for the presence of the causal agent of BGC proved negative.</li> </ul>
Bovine Tuberculosis (BT)*	<ul style="list-style-type: none"> <li>• donor animals without any sign of BT on the day of collection of the semen and either: kept in an AI center free from BT in a country, zone or compartment free from BT and which only accepts animals from free herds in a free country, zone or compartment; or negative results to tuberculin tests carried out annually and kept in a herd free from BT.</li> </ul>
Bovine Tuberculosis (BT) in farmed cervidae*	<ul style="list-style-type: none"> <li>• no sign of BT in any species on the day of collection of the semen; and either: herd free from BT in a country, zone or compartment free from BT of farmed cervidae, and which only accepts animals from free herds in a free country, zone or compartment; or negative results to tuberculin tests carried out annually and were kept in a herd free from BT.</li> </ul>
Blue Tongue Virus (BTV)	<ul style="list-style-type: none"> <li>• animals kept in a BTV free country or zone for at least 60 days before commencement of, and during, collection of the semen; or subjected to a serological test between 21 and 60 days after the last collection, with negative results; or subjected to an agent identification test on blood samples collected at commencement and conclusion of, and at least every 7 days (virus isolation test) or at least every 28 days (PCR test) during semen collection, with negative results.</li> </ul>
Bovine Viral Diarrhea (BVD)	<ul style="list-style-type: none"> <li>• animals subjected to a virus isolation test or a test for virus antigen, with negative results. Only when all the animals in pre-entry isolation have had negative results, may the animals enter the semen collection facilities;</li> <li>• animals subjected to a serological test to determine the presence or absence of BVD antibodies. Only if no seroconversion occurs in the animals which tested seronegative before entry into the pre-entry isolation facility, may any animal (seronegative or seropositive) be allowed entry into the semen collection facilities;</li> <li>• if seroconversion occurs, all the animals that remain seronegative should be kept in pre-entry isolation until there is no more seroconversion in the group for a period of 3 weeks. Serologically positive animals may be allowed entry into the semen collection facilities;</li> <li>• animals negative to previous serological tests should be retested to confirm absence of antibodies. Should an animal become serologically positive, every ejaculate of that animal collected since the last negative test should be either discarded or tested for virus with negative results.</li> </ul>
Contagious Bovine Pleuropneumonia (CBPP)*	<ul style="list-style-type: none"> <li>• from CBPP free countries, zones or compartments**: donor animals without clinical sign of CBPP on the day of collection of the semen; kept in a CBPP free country since birth or for at least the past 6 months;</li> <li>• from CBPP infected countries or zones: no clinical sign of CBPP on the day of collection of the semen; animals subjected to the CFT for CBPP with negative results, on two occasions (interval between each test from 21 to 30 days, the second test within 14 days prior to collection); isolated from other domestic bovidae from the day of the first CFT until collection; kept since birth, or for the past six months, in an establishment where no case of CBPP was reported during that period, and that the establishment was not situated in a CBPP infected zone; AND EITHER: not been vaccinated against CBPP; OR vaccinated using a vaccine complying with the standards described in the TM not more than 4 months prior to collection.</li> </ul>



Enzootic Bovine Leucosis (EBL)*	<ul style="list-style-type: none"> <li>• donor bull resident at the time of semen collection in an EBL free herd; and if less than 2 years of age, the bull came from a serologically negative ‘uterine’ dam; or bull subjected to diagnostic tests for EBL on blood samples on two occasions with negative results (first test at least 30 days before and the second test at least 90 days after semen collection).</li> </ul>
Foot and Mouth Disease (FMD)	<p>FMD free countries</p> <ul style="list-style-type: none"> <li>• with no clinical signs of FMD on the day of collection of semen and for the following 30 days;</li> <li>• animals kept for at least 3 months prior to collection in an FMD free country or zone without vaccination or a FMD free compartment;</li> </ul> <p>FMD infected countries</p> <ul style="list-style-type: none"> <li>• no clinical sign of FMD on the day of collection of the semen;</li> <li>• animals kept in an establishment where no animal had been added in the 30 days before collection, and that FMD has not occurred within 10 kilometers for the 30 days before and after collection;</li> <li>• have not been vaccinated and were subjected, not less than 21 days after collection of the semen, to tests for antibodies against FMD virus, with negative results; or had been vaccinated at least twice (last vaccination not more than 12 and not less than one month prior to collection);</li> <li>• no other animal present in the artificial insemination center has been vaccinated within the month prior to collection;</li> <li>• the semen subjected, with negative results, to a test for FMDV infection (if donor animal vaccinated within the 12 months prior to collection); stored in the country of origin for a period of at least one month following collection, and that during this period no animal on the establishment showed any sign of FMD.</li> </ul>
Infectious Bovine Rhinotracheitis/ Infectious Pustular Vulvovaginitis * (IBR-IPV)	<ul style="list-style-type: none"> <li>• Fresh semen: IBR/IPV free herd at the time of collection of the semen;</li> <li>• Frozen semen: IBR/IPV free herd at the time of collection of the semen; or donor animals in isolation during the period of collection and for the 30 days following collection and subjected to a diagnostic test for IBR/IPV on a blood sample taken at least 21 days after collection of the semen, with negative results; or if unknown serological status of the bull or positive serology, an aliquot of each semen collection subjected to a virus isolation test or PCR, performed in accordance with the TM, with negative results.</li> </ul>
Lumpy Skin Disease* (LSD; caused by group III virus, type Neethling)	<ul style="list-style-type: none"> <li>• from LSD free countries (cattle and water buffaloes): no clinical sign of LSD on the day of collection of the semen; kept for at least 28 days prior to collection in an LSD free country;</li> <li>• from countries considered infected with LSD: no clinical sign of LSD on the day of semen collection (SC) and for the following 28 days; kept in the exporting country for the 28 days prior to SC, in an establishment or AI center where no official case of LSD during that period, and establishment or AI center was not situated in an LSD infected zone; and either: vaccinated against LSD (28 to 90 days before SC and thereafter vaccinated annually); or tested with negative results using a serum neutralization test (SNT) or an indirect enzyme-linked immunosorbent assay (ELISA) for LSD on the day of first or up to 90 days after last SC; or stable seropositivity (not more than a two-fold rise in titre) on paired samples (tested side by side) to indirect ELISA or SNT carried out in quarantine, 28-60 days apart (first sample taken on the day of first SC).</li> </ul>
Trichomonosis*	<ul style="list-style-type: none"> <li>• donor animals never been used for natural service; or have only mated virgin heifers; or kept in an establishment or AI center where no case of trichomonosis has been reported; animals subjected to a direct microscopic and culture of preputial specimens with negative results.</li> </ul>

\*For each listed disease, Veterinary Authorities of importing countries should require the presentation of an international veterinary certificate attesting that the semen was collected, processed, and stored in conformity with the provisions of Chapters 4.5 and 4.6. \*\*Compartment means an animal subpopulation contained in one or more establishments under a common biosecurity management system with a distinct health status with respect to a specific disease or specific diseases for which required surveillance, control, and biosecurity measures have been applied for the purpose of international trade.

Table 5. Assessment of the sanitary risk for *in vivo* derived embryos using scientific approaches.

	<i>In vivo</i> infection	<i>In vitro</i> infection
<i>In vivo</i> embryo transfer	<ul style="list-style-type: none"> <li>naturally infected donor or insemination with infected semen;</li> <li>Embryos transferred to recipients.</li> </ul>	<ul style="list-style-type: none"> <li>experimentally spiked embryos/semen;</li> <li>embryos transferred to recipients.</li> </ul>
<i>In vitro</i> embryo washing	<ul style="list-style-type: none"> <li>naturally infected donor or insemination with infected semen;</li> <li>embryo status analyzed after washing procedure described in IETS Manual.</li> </ul>	<ul style="list-style-type: none"> <li>experimentally spiked embryos/semen;</li> <li>embryo status analyzed after washing procedure in IETS Manual.</li> </ul>

Concerning transmission risk via ET, the IETS HASAC Committee reviews scientific publications on an annual basis and updates a complete set of more than 400 references, which can be consulted on their website ([www.iets.org](http://www.iets.org)). In the bovine species, 89 potential embryo pathogens have been investigated (Thibier, 2011). All diseases and pathogenic agents have been placed into one of four categories based on the amount of research indicating the likelihood of disease control through the use of embryo transfer (Table 6). For category 1 diseases, risk of transmission of a given disease from donor to recipient via an embryo is negligible, providing biosecurity measures described for handling embryos, material disinfection, and animal health requirements (semen, donor, and recipients) as described in the IETS Manual have been respected (Stringfellow, 2010; Thibier, 2011).

New questions have been raised regarding trypsin treatments: Al Ahmad *et al.* (2012) compared a treatment standard (TS) comprised of phosphate-buffered saline (PBS), 0.4% BSA (five washes of 100 fold dilution for 10 sec each), followed by two treatments with 0.25% trypsin in Hank's solution (45 sec each), and then PBS 0.4% BSA again (five times for 10 sec). The four other washing procedures all included the same first and last washing steps with PBS but without BSA (five times for 10 sec) and with PBS 0.4% BSA (five times for 10 sec), respectively. The intermediate step varied for each washing procedure, with other trypsin treatments (longer time, twice for 60 sec) or hyaluronidase treatments in order to eliminate Blue tongue virus (BTV) from *in vitro* infected goat embryos: only two trypsin treatments of 60 sec each was effective in removing BTV from the embryos.

#### *Legal and sanitary measures applied to in vivo derived embryos*

Practical guidelines have been published in the Manual of the International Embryo Transfer Society in order to provide risk management procedures ensuring the safety of ET (Stringfellow, 2010). Since these guidelines have been adopted by the OIE, they are well accepted and implemented worldwide (OIE, 2012; Chapter 4.7). In Europe, legislation prescribes the sanitary conditions to which embryo collection and transfer should comply. The Council Directive

89/556/EEC of 25 September 1989 describes animal health conditions governing intra-community trade in and importation from third countries of embryos derived from the bovine species. The legislation defines sanitary and biosecurity requirements including donor females, environmental and handling conditions, and semen used for donor insemination.

- Sanitary requirements

In addition to OIE recommendations (Table 7), EU legislation includes the following requirements: donor cows must have spent the previous 6 months within community territory or in the third country of collection in a herd officially tuberculosis and brucellosis free, enzootic bovine leucosis free (or no clinical signs of enzootic bovine leucosis during the previous 3 years); and where no clinical signs of infectious bovine rhinotracheitis/infectious pustular vulvo-vaginitis have been observed during the previous year.

- Environmental and handling conditions

Both the OIE Terrestrial Code (Chapter 4.7) and Directive 89/556 include biosecurity measures based on a team approved by competent authority (government or local veterinary authorities), supervised by a team veterinarian responsible for all team operations (health status of donor cows, appropriate disease control measures with handling and operating on donors, disinfection, and hygiene procedures). Team personnel should be adequately trained in the techniques and principles of disease control. High standards of hygiene should be practiced to preclude the introduction of disease.

Procedures, facilities, and equipment are verified through regular official inspections (at least once a year) regarding embryo collection, process, and manipulation of embryos at a permanent site or mobile laboratory, storage of embryos as well as activity records.

The testing of samples can be requested by an importing country to confirm the absence of pathogenic organisms that may be transmitted via *in vivo* derived embryos (see Table 9), or to assess the quality control of the collection team together with washing procedures. Specimens may include degenerated embryos, embryo collection fluids, and a pool of the last washes of the embryos. In the French regulations, this testing procedure is performed annually in a central laboratory



and represents a prerequisite of renewal of approval together with a favorable report from the official inspection (France, 2008).

- Sanitary controls of semen used in embryo transfer

The safety of semen is another critical point and international regulations include requirements regarding ejaculates being used for assisted reproduction techniques (Wrathall *et al.*, 2006). With regard to semen that is used to

produce embryos for international trade, batches of frozen semen are selected from bulls located in accredited AI Centers in the majority of cases. Such bulls are normally certified negative for acute, epidemic diseases such as foot-and-mouth disease, and chronic diseases such as brucellosis, tuberculosis, leptospirosis, campylobacteriosis, and trichomonosis. For international trade, some countries request that bulls are certified negative for enzootic bovine leukosis, infectious bovine rhinotracheitis, and bovine viral diarrhea. (Bielanski, 2006; Wrathall *et al.*, 2006).

Table 6. Diseases or infectious agents in cattle listed by IETS according to the risk for their transmission via *in vivo* derived embryos (OIE, 2012).

Disease category	Disease agent
Category 1: Sufficient evidence has accrued to show that the risk of transmission is negligible provided that the embryos are properly handled between collection and transfer according to the IETS Manual.	Bluetongue Bovine spongiform encephalopathy <i>Brucella abortus</i> Enzootic bovine leukosis Foot and mouth disease Infectious bovine rhinotracheitis: trypsin treatment required
Category 2: Substantial evidence has accrued to show that the risk of transmission is negligible provided that the embryos are properly handled between collection and transfer according to the IETS Manual, but for which additional transfers are required to verify existing data.	None
Category 3: Preliminary evidence indicates that the risk of transmission is negligible provided that the embryos are properly handled between collection and transfer according to the IETS Manual, but for which additional <i>in vitro</i> and <i>in vivo</i> experimental data are required to substantiate the preliminary findings.	Bovine immunodeficiency virus Bovine viral diarrhea virus Rinderpest virus <i>Campylobacter fetus (subs. venerealis)</i> <i>Haemophilus somnus</i> <i>Mycobacterium paratuberculosis</i> <i>Neospora caninum</i>
Category 4: No conclusions are yet possible with regard to the level of transmission risk, or the risk of transmission via embryo transfer might not be negligible even if the embryos are properly handled according to the IETS Manual between collection and transfer.	Akabane Bovine anaplasmosis Bovine herpesvirus-4 Enterovirus Lumpy skin disease Vesicular stomatitis <i>Chlamydia psittaci</i> <i>Escherichia coli 09:K99</i> <i>Leptospira borgpetersenii serovar hardjobovis</i> <i>Mycobacterium bovis</i> Parainfluenza-3 virus <i>Trichomonas foetus</i> <i>Ureaplasma and Mycoplasma spp.</i>

*Requirements applicable to in vitro produced (IVP) embryos*

*Assessment of disease transmission via in vitro produced embryos*

*In vitro* embryo production entails the completion of three biological steps that are now well

established in cattle: oocyte maturation, *in vitro* fertilization (IVF), and embryo culture. The following factors have hindered progress toward the establishment of recognized sanitary procedures for IVP embryos.

The zona pellucida of intrafollicular oocytes appears to differ from that of ovulated ova. This structural difference might be associated with differing resistance to adherence to or penetration of the zona pellucida by



infectious agents (Marquant-LeGuienne *et al.*, 2010). Thus, simple extrapolation from sanitary procedures described for *in vivo* derived embryos is not advised.

Oocytes may be collected either from ovaries of slaughtered animals or by ovum pick-up, which involves ultrasound-guided transvaginal aspiration of oocytes from ovarian follicles. In the first instance, the sanitary status of the slaughtered females is not well defined, which increases sanitary risks associated with IVP. In the case of ovum pick-up, control of the sanitary

status of the donor cow is greater (Table 7). Regardless of the source of oocytes, one should consider incorporating special precautions into protocols for IVP of embryos. Any biological product used in the recovery of gametes, sperm, and oocytes or embryos, dilution, *in vitro* maturation of oocytes, and washing or storage is potentially a source of contamination. Indeed, contamination of slaughterhouse oocytes with BVDV and BHV-1 has been reported (Marquant-LeGuienne *et al.*, 2000; Galik *et al.*, 2002).

Table 7. Potential sources of pathogen transmission related to *in vitro* embryo production.

Origin	Slaughterhouse oocytes	OPU oocytes
Donor, Ovaries, Oocytes	<ul style="list-style-type: none"> <li>randomly collected ovaries;</li> <li>unknown health status of the donor animals (risk of clinical or subclinical diseases);</li> <li>pool of oocytes during transportation to the IVF laboratory, and then during IVM, IVF, and IVC treatment.</li> </ul>	<ul style="list-style-type: none"> <li>ovaries are collected from ovaries of well identified animals;</li> <li>health status of donor females well known;</li> <li>oocytes can easily be treated separately if necessary.</li> </ul>
Semen	<ul style="list-style-type: none"> <li>sperm fraction used for <i>in vitro</i> fertilization (seminal plasma removal via different methods as “swim up” or Percoll gradient centrifugations);</li> <li>use of cryopreserved spermatozoa to achieve a high rate of fertilization.</li> </ul>	
Environment, Media	<ul style="list-style-type: none"> <li>pathogens present in serum or media (containing animal derived products, use of cell lines) used for the <i>in vitro</i> maturation (IMV), fertilization (IVF), culture or handling of embryos;</li> <li>added during the manipulation of embryos (collection of the oocytes, washing, culture, or transfer of embryos).</li> </ul>	

Moreover, risk assessment should not be extrapolated from *in vivo* to *in vitro* produced embryos, or from one pathogen to another (Thibier, 2011). This was illustrated by Bielanski *et al.* (2009) in an experiment comparing two BVDV biotypes (NY-1 vs. PA-131) added to bovine IVP embryos, treated according to IETS recommendations and then transferred to recipients (Table 8). The proportion of seroconverted recipients differed between the two viruses. Even in the “worst-case” strain, term pregnancies resulted in seronegative calves, demonstrating that risk of disease transmission to offspring and recipients remains low. Another recent experiment reported that approximately 20% of

embryos still remained infected following the IETS-recommended 10-sequential wash procedure, after exposure *in vitro* to BVDV type 2 (strain PA-131; Lalonde and Bielanski, 2011).

Thus, the following suggestions have been made in France regarding the prevention of contamination of IVP embryos from the donor side (Fig. 2) as well as environment and media: washings of IVP embryos (IETS recommendations), addition of synthetic compounds in the media, use of controlled cell lines, certification for companies and products, re-testing biological products before use (mainly for BVDV and *Mycoplasma* sp.), and media heating (56°C/30 min).

Table 8. Comparison of subtypes of bovine viral diarrhea virus with *in vitro*-produced embryos (Bielanski *et al.*, 2009).

Type of non cytopathic BVDV	NY-1	PA-131
Number of pregnancies/number of transfers	20/33	25/61
Percentage of seroconversions in recipients	0%	51.4%
Number of seroconversions in offspring	0 (18 full-term calves)	0 (2 full-term calves)
Virus isolation tests on non-transferred embryos (%)	25%	28%

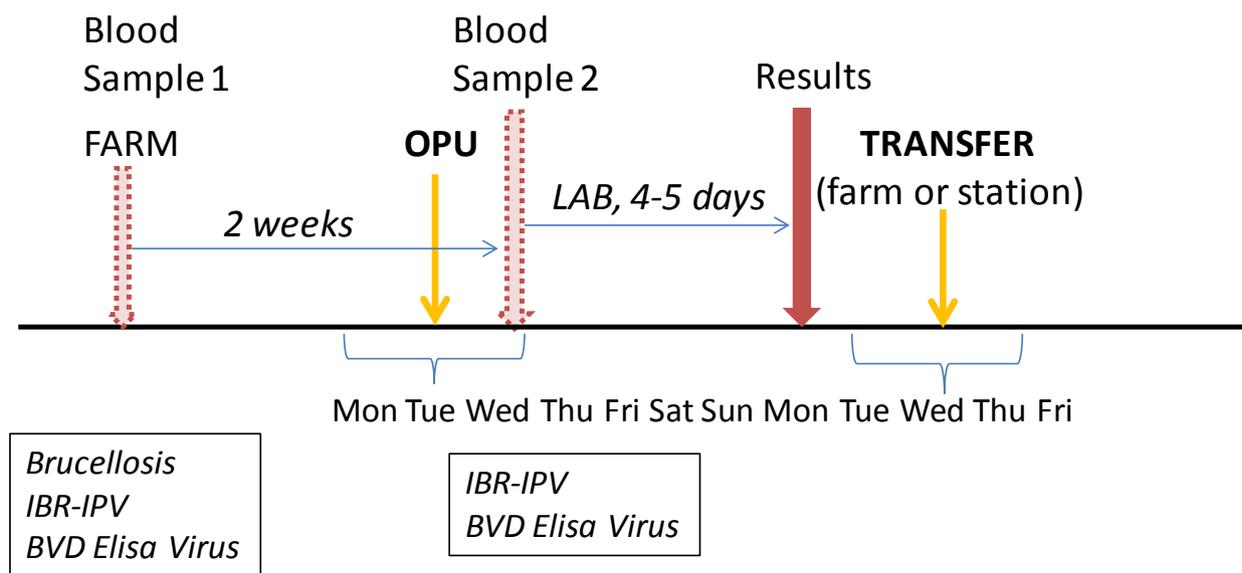


Figure 2. Additional sanitary controls recommended in France and voluntarily applied to donor cows before Ovum Pick Up (OPU) on farms.

*Legal and sanitary measures applied to in vitro produced embryos*

Practical guidelines have been published in the Manual of the International Embryo Transfer Society (IETS) in order to provide risk management procedures ensuring the safety of herds using IVP (Marquant-LeGuienne *et al.*, 2010). Ideally, quality-assurance procedures should be outlined for buildings, staff, biological materials, and *in vitro* manipulations. The general plan of quality assurance should include adequate documentation including general procedures, operating modes with details of specific laboratory procedures and traceability documents (Marquant-LeGuienne *et al.*, 2010). The international and national legislations define sanitary and biosecurity requirements including donor females, environmental and handling conditions, and semen used for donor insemination.

Since adopted by the OIE, biosecurity measures have been implemented and accepted

worldwide (OIE, 2012; Chapter 4.7). According to these recommendations, embryos should be produced by a team approved by a national sanitary authority and under supervision of a team veterinarian. When oocytes are collected from ovaries of slaughtered animals, the slaughterhouse should be inspected regularly by official veterinary authorities. In addition, IVP embryos should be washed using techniques shown to be effective for *in vivo*-derived embryos in the IETS Manual. As in the case of *in vivo* derived embryos, donor cow status is described in the Terrestrial Code, which distinguishes clearly between recovering oocytes from live donors and from slaughterhouse ovaries (Table 9; OIE, 2012; Chapter 4.8). In Europe, legislation prescribes the sanitary conditions to which embryo collection and transfer should comply. The Council Directive 89/556/EEC of 25 September 1989 describes animal health conditions governing intra-community trade in and importation from third countries of IVP embryos of the bovine species.



Table 9. Diseases specific OIE recommendations for the importation of bovine embryos (OIE, 2012).

Disease*	<i>In vivo</i> derived embryos	<i>In vitro</i> produced embryos/ova
Bovine Brucellosis (BB)*		<ul style="list-style-type: none"> <li>donor females kept in a country or zone free from BB; or kept in a herd officially free from BB (tests as prescribed in Chapter 1.3); oocytes fertilized with semen meeting the conditions referred to in Chapters 4.5 and 4.6.</li> </ul>
Blue Tongue Virus (BTV)		<ul style="list-style-type: none"> <li>donor cows kept in a BTV free country or in a seasonally free zone or in a vector-protected establishment (in BTV infected countries) for at least the 60 days prior to, and at the time of, collection of the embryos;</li> <li>donor cows subjected to a serological test between 21 and 60 days after collection, with negative results;</li> <li>donor cows subjected to an agent identification test taken on the day of collection, with negative results.</li> </ul>
Foot and Mouth Disease (FMD)		<p>FMD free countries</p> <ul style="list-style-type: none"> <li>no clinical sign of FMD at the time of collection of the oocytes;</li> <li>donor kept at the time of collection in a FMD free country or zone with or without vaccination or a FMD free compartment;</li> <li>embryos produced in zones with vaccination and destined for an FMD free country or zone without vaccination or an FMD free compartment: no vaccination of donor and negative results to tests for antibodies against FMD virus; or vaccinated at least twice (last vaccination not less than one month and not more than 12 months prior to collection); no other animal present in the establishment vaccinated within the month prior to collection.</li> </ul>
Bovine Tuberculosis (BT) in cattle or farmed cervidae*	<ul style="list-style-type: none"> <li>no sign of BT during the 24 h prior to embryo collection in the herd of origin; and either donor originated from a herd free from BT (cattle or farmed cervidae) in a country, zone or compartment free from BT; or kept in a herd free from BT (cattle or farmed cervidae), and subjected to a tuberculin test for BT with negative results during an isolation period (30 days) in the establishment of origin prior to collection (COL).</li> </ul>	
Contagious Bovine Pleuropneumonia (CBPP)*	<ul style="list-style-type: none"> <li>from CBPP free countries, zones or compartments: donor animals without clinical sign of CBPP on the day of collection of the embryos/oocytes; kept in a CBPP free country since birth or for at least the past 6 months; oocytes fertilized with semen meeting the conditions of Article 11.8.8;</li> <li>from CBPP infected countries or zones: no clinical sign of CBPP on the day of COL of the embryos/oocytes; donor subjected to the CFT for CBPP with negative results, on two occasions (21 to 30 days between each test, 2nd test within 14 days prior to COL); isolated from other domestic bovidae from the day of the first CFT until COL; kept since birth, or for the past 6 months, in an establishment where no case of CBPP was reported, and that the establishment was not situated in a CBPP infected zone; AND EITHER: not been vaccinated against CBPP; OR vaccinated using a vaccine complying with the standards described in the TM not more than 4 months prior to COL; oocytes fertilized with semen meeting the conditions of Article 11.8.9.</li> </ul>	
Lumpy Skin Disease (LSD; caused by group III virus, type Neethling)*	<ul style="list-style-type: none"> <li>from LSD free countries (embryos/oocytes of cattle and water buffaloes): donor animals without clinical sign of LSD on the day of COL of the embryos/oocytes;</li> <li>from countries considered infected with LSD (embryos/oocytes of cattle and water buffaloes): no case of LSD has been reported during the 28 days prior to COL in the establishment; and no clinical sign of LSD on the day of COL; and either: vaccinated against LSD between 28 days and 90 days before first embryo/oocyte COL and thereafter vaccinated annually; or tested with negative serological results (SNT or indirect ELISA) for LSD on the day of embryo/oocyte COL or up to 90 days after last collection; or showed stable seropositivity on paired samples tested side by side to indirect ELISA or SNT carried out in quarantine (28–60 days apart, one sample on the day of COL).</li> </ul>	

\*For each listed disease as well as Enzootic Bovine Leucosis (EBL) and Infectious Bovine Rhinotracheitis/Infectious Pustular Vulvovaginitis (IBR-IPV), Veterinary Authorities of importing countries should require the presentation of an international veterinary certificate attesting that the embryos/ova have been collected, processed, and stored in conformity with the provisions of OIE Chapters 4.7, 4.8, and 4.9.



## Conclusion

Since 40 years, billions of embryos and semen straws have been distributed around the world. This fact is reassuring that reports implicating germplasm in disease transmission are extremely rare. The high degree of biosecurity measures under official approval and the professionalism of ET teams and the good practices of the AI industry ensures germplasm movement with negligible risk of disease transmission using gametes and embryo based biotechnologies. While emerging diseases threaten international trade, increased use of *in vitro* embryo production and micromanipulation and pre-implantation diagnoses necessitate the updating of specific guidelines and related research work.

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## Genomic selection and assisted reproduction technologies to foster cattle breeding

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### Abstract

Genomics has been propagated as a “paradigm shifting” innovation in livestock during the last decade. The possibility of predicting breeding values using genomic information has revolutionized the dairy cattle industry and is now being implemented in beef cattle. In this paper we discuss how genomics is changing cattle breeding through genomic selection, and how this change is creating new ways to articulate assisted reproduction technologies with animal breeding. We also debate that the scientific community is still starting the long journey to reveal the functional aspects of the cattle genome, and that knowledge in this field is the frontier to a whole new venue for the development of novel applications in the livestock sector.

**Keywords:** breeding, cattle, genomic selection, reproductive biotechnologies.

### How genomics is changing cattle breeding

Since the first initiatives to sequence the human genome made their data publically available back in 2001 (International Human Genome Sequencing Consortium *et al.*, 2001; Venter *et al.*, 2001), scientists have been looking for sustainable ways to apply genomic information in several areas of human activity. The community quickly realized that the early developments of this pursuit had great potential to be applied to the livestock sector due to its economic importance, which led to the prioritization of livestock as a major target for developing real world applications of genomics. In fact, this trend started to become reality in 2002, when the Bovine Genome Project was conceived and implemented. Seven years later, as one of the major achievements in livestock genomics, the conclusion of the *Bos primigenius taurus* genome in 2009 (Bovine Genome Sequencing and Analysis Consortium *et al.*, 2009) opened the possibility for the development of a range of analytical tools capable to help on the exploration of the genomic features of the *Bos* genus (Van Tassell *et al.*, 2008; Matukumalli *et al.*, 2009; Boichard *et al.*, 2012).

Among these genomic analytical tools, one of the most popular is commonly called “genomic chip”.

With these devices, it is possible to analyze up to few millions of genetic markers, known as single nucleotide polymorphism (SNP), from a single DNA sample. Thanks to the existence of the cow reference genome, any genetic marker can be easily linked to a chromosomal location, enabling to perform a multitude of association statistical tests to help in the integration of chromosomal coordinates, SNP markers and phenotypes, and enabling the development of applications in the livestock breeding sector.

In a very fundamental paper, written almost a decade before the completion of the cow genome and the launch of the first cattle “genomic chip”, Meuwissen *et al.* (2001) proposed that selection on estimated breeding values (EBVs) predicted from genetic markers could substantially increase the rate of genetic gain in animals and plants, especially if combined with reproductive techniques to shorten the generation interval. Although their analyses were performed using simulated DNA marker data, since no real data was available at that time, the possibility of predicting the genetic merit of an individual using genomic information associated with pedigree and phenotypic databases influenced the entire livestock genetics community and objective steps were taken in order to transform their idea in reality.

Later, with the concrete perspective of the cow genome conclusion and the imminent development of the first “genomic chip” specific for cattle, Schaeffer (2006) proposed that animals could be genotyped for thousands of SNPs located at roughly one centimorgan (1 cM) intervals throughout the genome, so that a “genomic” estimated breeding value (gEBV) could be determined if the effects of each analyzed SNP could be estimated. He also argued that, since the eventual gEBV could be calculated at birth, it should be possible to compare this new method with the traditional progeny testing strategy used in dairy cattle. His expectations at that time were that costs associated with proving bulls could be reduced by 92% and genetic change increased by a factor of 2. He proposed that genome-wide selection could become a popular tool for genetic improvement in livestock.

The original predictions of Meuwissen *et al.* (2001) and Schaeffer (2006) were shown to be reasonably accurate just after the first cattle “genomic

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chip” came to the market. The first commercially available bovine “genomic chip” was the so-called “BovineSNP50” (developed by the company Illumina Inc. in collaboration with the United States Department of Agriculture - USDA), being considered as a robust platform for mapping disease genes and quantitative trait loci (QTL) in cattle (Matukumalli *et al.*, 2009). In fact, this genomic tool was the basis for the development of genomic selection in dairy cattle, one of the most elegant and successful applications of genomics in livestock up to date.

In order to improve genomic analysis in cattle, new “genomic chips” with different marker densities and configurations were developed recently, enabling better research achievements and making it real the routine use of genomics for breeding purposes in cattle (Boichard *et al.*, 2012). Nowadays, there are “genomic chips” available for the major livestock species in different formats and marker densities, so it is expected that there will be an explosion of discoveries and applications in the livestock sector over the next years due to these tools.

#### **Genomic selection as a tool to refine breeding strategies**

It is safe to affirm that the only economically sustainable large application of genomics in livestock so far is the dairy cattle genomic selection initiative, more particularly in Holstein breeding programs. Trying to reduce the high costs associated with progeny tests, and in order to increase the velocity of genetic improvement in the Holstein breed from USA and Canada, artificial insemination and pharmaceutical companies joined forces in a consortium with research institutions from these countries in order to put in practice the ideas of Meuwissen *et al.* (2001). As the result of this initiative, initiated in 2004, the first bovine “genomic chip” was launched in 2007 and the first experimental genomic evaluation was performed in USA/Canada in 2008. Since 2009, genomic predictions are part of the Holstein Association routine services to dairy breeders (Scheffer and Weigel, 2012).

Genomic selection conceptually consists in the use of pre-existent pedigree and phenotypic records, associated with genomic information to generate gEBVs. In contrast with the traditional EBVs, the new methodology provides indexes with higher reliabilities. Table 1 illustrates the extent of the improvement in reliability rates by comparing traditional and genomic EBVs in dairy cattle phenotypes (in this case LPI, an index related to the profit offered by the cow during its entire life). A two-fold increase in the average reliability has doubled the genetic change rate in Holstein breeding programs as predicted by Schaeffer (2006). Additionally, the genomic identification of the best calves in early stages of life, who will be kept as young bull candidates for the progeny tests, has reduced the

maintenance costs and the generation interval as suggested by Meuwissen *et al.* (2001), representing the best justification for the massive application of genomic selection we witnessed in the recent years. Other traditional dairy breeds from North America, such as Jersey, Ayrshire and Brown Swiss are taking advantage of this technology by applying genomic selection in their breeding processes since 2009.

Diversely, the beef cattle sector has been struggling with the attempt to apply genomics in its genetic evaluation routine for years, with relatively less success. The first experiences were made using SNP panels containing low number of genetic markers (between 96 and 384 SNPs) and commercialized mainly by companies traditionally dedicated to livestock health and reproduction areas. Those pioneer initiatives were originated from the idea that the use of few SNP markers, associated with genes related to phenotypes of interest, would allow breeders to test a given animal and make strategic decisions.

In North America, this type of genomic test is still being applied to some beef breeds, in special to Angus (Gill *et al.*, 2009; Garrick, 2011), using a “blending” approach as the way to improve EBV’s reliability. Other breeds, such as the dual-purpose Simmental and Braunvieh Schweiz (Brown Swiss in Europe), are implementing the genomic selection protocols similarly as in dairy breeds, although still in a very preliminary way (Gredler *et al.*, 2009; Croiseau *et al.*, 2012)

In Brazil, since 2005 and specifically for Nelore (the major beef breed in the country), there were several frustrated attempts to implement genomics in breeding and selection. Several factors have concurred to the failure of implementing this type of initiative, but three of them were of capital importance and should be closely analyzed. The first one was the fact that in the beginning, the first SNP tests were not integrated to the traditional breeding programs, either because their costs were prohibitive or the breeders could not assess the benefits of investing in that new technology. Based on the market strategies and technical data presented at that time, the final user (the breeder) was not able to know how the investment would be recovered since the promised increase in reliability did not provide enough parameters for proper economic calculations. Another important factor was that the business model of those companies did not comprehend the sharing of real data information (SNP variants and its effects) to the breeders, offering results in a “score-type system” which breeders should use as the selection criteria. Finally, that approach was overcome by the genomic selection concept, widely applied in dairy breeds from 2008, and where instead of couple hundreds, several thousands of SNP effects are calculated and used to infer the genetic value of an individual.

Nowadays, several research groups are depositing their efforts on the development of new low



density “genomic chips” (containing between three and twenty thousand SNPs) to be routinely used in beef cattle genomic selection programs. The idea behind this strategy is to create a DNA test that could reach reasonable price and high precision simultaneously, serving to the purposes and peculiarities of beef breeding systems. The use of inference methods to generate high density genotypes from low density ones, known as imputation, is the key for its success, allowing for

reduction in genotyping associated costs while maintaining the quality of genomic predictions. However, there are several unsolved expectations still to be clarified about how the beef sector will fully benefit from genomics, as the dairy sector did recently. The costs associated with DNA testing is one of the most striking factors, since in beef cattle (diversely from dairy), the unitary value of an individual makes prohibitive to test a large number of animals in a herd.

Table 1. Average gain in LPI (Lifetime Profit Index) reliability due to genomics in Canadian Holstein - August 2013.

Sub-group for Holstein breed	Average LPI Reliability (%)			
	Traditional	Genomics <sup>a</sup>	Gain	DGV Weight <sup>b</sup>
50k <sup>c</sup> Young bulls and heifers (born 2010-2012)	38	70	32	65%
LD <sup>d</sup> (3k or 6k) heifers (born 2011-2013)	34	67	33	66%
LD <sup>d</sup> Younger hows in 1 <sup>st</sup> or 2 <sup>nd</sup> lactation	50	69	19	58%
LD <sup>d</sup> Foreign hows with MACE <sup>c</sup> in Canada	42	69	27	63%
1st Crop proven sires in Canada	85	90	5	51%
Foreign sires with MACE <sup>c</sup> in Canada	69	83	14	55%

<sup>a</sup>Evaluation based on the Genomic Parent Average (GPA), which combines the Parent Average (PA) with the Direct Genomic Value (DGV). <sup>b</sup>Weight attributed to the DGV in order to combine PA and DGV. <sup>c</sup>Animals genotyped with Illumina® BovineSNP50 Genotyping BeadChip (a.k.a. 50k), which interrogates 54,609 SNPs. <sup>d</sup>Animals genotyped either with Illumina® BovineLD Genotyping BeadChip (a.k.a. LD or 6k) or Illumina® GoldenGate® Bovine3k Genotyping BeadChip (a.k.a. 3k), which interrogates 6,909 and 2,900 SNPs, respectively. <sup>e</sup>Multiple-trait Across Country Evaluations. Adapted from Canadian Dairy Network <<http://www.cdn.ca/articles.php>>.

### Articulating genomic selection and assisted reproduction technologies

For decades, genetic evaluations (GE) and assisted reproduction technologies (ART) have been tracking different scientific pathways. However, in the context of application, GE and ART have been working together to promote genetic changes in commercial herds. While breeding programs use mass selection approaches for GE to identify phenotypically superior animals, considering several traits simultaneously and weighting them in selection indexes based in EBV with high reliabilities, ART such as artificial insemination, embryo *in vitro* production, and embryo transfer, have been applied to the multiplication of animals considered to be superior. Genomic predictions now offer a new opportunity for using ART to shorten generation intervals and maximize genetic gain, in special the use of preimplantation genetic diagnosis (PGD).

The commercial use of PGD has been limited not only by technical restraints such as training technicians in embryo micromanipulation and embryo biopsy procedures, but also by the existence of a small number of genetic tests with justifiable benefit, low cost, and commercial potential available. Perhaps the largest present application of PGD is embryo sexing, which allows for the identification of male and female embryos prior to transfer to a recipient cow. However, semen sex

sorting is being rapidly adopted as a replacing technology to embryo sexing in the cattle industry.

As the genome of the embryo remains unchanged in the somatic cells of the adult, PGD can be used as a satellite technology to speed up genetic gain using genomic selection. In this context, an embryo biopsy can be performed, its DNA extracted, and a “genomic chip” used to estimate the gEBV of an animal that was never born. One may want to transfer only embryos exhibiting satisfactory gEBVs, and discard the others. Similarly, pregnancy can be marketed with fetuses with known and quantified genetic merit.

Lauri *et al.* (2013) have proved the concept that faithful genotypes can be retrieved from amplified DNA of embryo biopsies by using samples of cloned embryos. However, the quality of the genotypes obtained is sensitive to the number of embryonic cells biopsied, i.e., to the initial amount of DNA available for amplification (Alonso *et al.*, 2013; FMVZ/USP, São Paulo, SP; unpublished data). Satisfactory results can be obtained from a typical embryo biopsy, and the quality of the genotypes can be substantially improved if genotypes of the sire and dam are available to be compared with the genotypes of the embryo. Embryo genotyping has already been used in reproductive programs of dairy breeds in Europe (Le Bourhis *et al.*, 2011) and North America (Sargolzaei *et al.*, 2013), and will soon be available for commercial application with



*in vitro* produced bovine embryos in Brazil.

### Next frontier in cattle genomics

Although the current development of genomic selection is a promising field allowing for broader applications in dairy and beef cattle, the generation of large amount of genomic data (either SNP or DNA sequence information) has the potential to create completely new lines of research, and take us beyond using whole genome information to predict breeding values. It is gradually becoming clear that exploiting the functional meaning of the genes underlying economically important traits can generate invaluable knowledge to develop new technologies.

Using SNP data, it is now possible to scan whole cattle genomes, and look for signals of associations between chromosome coordinates and traits of interest (Bush and Moore, 2012), or even seek genomic regions where past selection has taken place (Oleksyk *et al.*, 2010). By extracting functional information about the genes located nearby these signals, it is possible to give rise to a handful of interesting hypotheses on the biological pathways that underlie economically important traits. This is the revolution of the “hypothesis generating research”, which allows for setting experiments to elucidate physiological and pathological roles in phenotype differences based on findings from large scale genomics studies.

Studies taping the genomes of Brazilian cattle are only now emerging. For instance, a genome-wide scan for birth weight in Nelore cattle was recently performed and led to the discovery of a strong signal pointing out to a region in chromosome 14, which harbors a series of genes previously found to affect human and cattle stature (Utsunomiya *et al.*, 2013a). Another study looking for evidence of breed-specific recent selection detected genetic variations that may have been shaped by human-driven selection in four different cattle breeds (Utsunomiya *et al.*, 2013b). Among these, the dairy Gyr breed presented a signal mapping to the *ST6GALNAC5* gene, which is known to participate in the deposition of gangliosides in milk fat globules. These studies represent important resources for characterizing genome regions that affect traits of economic interest in Brazilian cattle.

### Conclusions

Analyzing the large amount of scientific literature related to cattle genomics produced in the past five years, it is possible to observe the extremely fast pace on the adoption of scientific knowledge in the practical daily applications. With this in perspective, it is feasible to postulate that in the near future the ART and GE approaches combined will be the driving forces to lead cattle breeding to a finer process than it is nowadays.

From one side, GE improved methods will make possible to know which gene alleles are the exact ones desired for a given type of animal. On the other hand, ART will allow to check the presence of these favorable alleles in early stage *in vitro* produced embryos, making the whole selection and breeding process extremely more accurate.

We also foresee the development of specific “genomic-audited” lineages, carrying specific and interconnected alleles selected inside the traditional breeds, which would offer better chances to the livestock industry to produce the animal required for each type of application.

This vision can also be beneficial to tropical animal production systems where traits related to environment adaptation (such as heat tolerance, low quality forage grazing ability, disease challenge resistance) play fundamental roles for its development, although still having their physiological basis to be uncovered.

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## Strategies to increase in vitro embryo yield: lessons from cell and molecular research

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### Abstract

Numerous reproductive biotechnologies are commonly employed to enhance animal production mainly through multiplying animals with high-quality traits in a large-scale production system. There is, however, several peculiarities during the process of embryo in vitro production that are still in need of further studies in order to obtain a higher efficiency. This present review discuss some of such particularities, as well as new models of embryo and gamete production, which will probably be part of a new era of reproductive biotechnologies in a near future.

**Keywords:** animal reproduction, bovine, embryo, *in vitro* studies.

### Introduction

The development of embryo technologies have been for long time challenging technicians to find better strategies and apply them in an efficient and low-cost manner, aiming benefits to animal production. Having such opportunities in mind, it is due to our society to face the challenges of improving such technologies, and also, to be ready and able to develop and adapt new technologies.

Last decades have witnessed a huge development of biotechnology methodologies, elaborated or else adapted for our specific conditions, such as embryo transfer (ET), *in vitro* fertilization (IVF), fixed time embryo transfer (FTET), and also some cryopreservation approaches that allow embryo large-scale production.

Theoretically, an efficient embryo production leads to a multiplying process of specifically selected animals and consequently, to an improvement on animal breeding. However such improvement is still not fully described in scientific reports or studies containing clear and evident examples.

Which would be the reasons? Would we have reached a plentiful competence so that such studies will not have to be performed? Are there still opportunities for the improvement of gamete generation or embryo production derived from selected animals?

### Oocyte donors

One of the most prominent properties in the process of embryo production is the individual variation

between donors. Such feature is highly noticeable and observed in both embryo or cumulus-oocytes complexes (COCs) recovery by superovulation/embryo transfer or ovum pick-up (OPU) procedures (Yang *et al.*, 2008; Pontes *et al.*, 2011).

The number of embryos produced by OPU/IVF is associated only to folliculogenesis and independent from uterine-environment or males, allowing therefore a very unbiased research strategy.

Indeed, it has been reported that oocyte recovery from some specific animals may be higher when compared to other animals, with a distribution similar to a Gaussian curve. Moreover, there is a tendency for such higher recovery to be maintained throughout several follicle aspirations sections (Garcia and Salaheddine, 1998). There are also reports showing that the pool of preantral follicles in a bovine ovary presents high correlation with the number of antral follicles found, and most importantly, with its fertility (Mossa *et al.*, 2012). Finally, discussions with colleagues that use OPU in a routine manner leads to the hypothesis that such characteristic is genetically transferred and that daughters of females that present a pool of increased antral follicles frequently present the same characteristics.

A study to observe such genetic characteristic was recently developed aiming the identification of molecular markers related to the number of viable COCs retrieved (Santos-Biase *et al.*, 2012). Even though few markers were evaluated, they have showed significant effect on COCs production, and 1.9 more COCs could be retrieved per OPU routine. Interestingly, the markers were present more frequently in zebu animals, and therefore may be associated to a higher OPU/IVF efficiency in these species. Therefore, studies applying large-scale embryo *in vitro* production systems are encouraged and may contribute to the increase of embryo production.

### Oocytes transcripts and competence

The oocyte competence to go through fecundation and to properly develop into blastocyst and term strongly depends on the synthesis and storage of several components during the oogenesis (Lonergan *et al.*, 2003; Gandolfi *et al.*, 2005; Sirard *et al.*, 2006). It is well established that such components (i.e. RNAs, proteins, and energetic substrates) are essential during

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the earliest stages of embryo development, when their transcriptional activity is limited (Memili *et al.*, 1998; Picton *et al.*, 1998; Memili and First, 2000; Meirelles *et al.*, 2004). In oocytes, gene expression is regulated and variable throughout oogenesis. Differentially expressed genes have been characterized in oocytes with greater developmental competence (Pan *et al.*, 2005; Fair *et al.*, 2007; Katz-Jaffe *et al.*, 2009; Mamo *et al.*, 2011). These findings support the hypothesis that specific mRNAs and proteins produced during oogenesis drive the adequate embryo development (Robert *et al.*, 2000; Fair *et al.*, 2004; Gutiérrez-Adán *et al.*, 2004; Meirelles *et al.*, 2004; Mourot *et al.*, 2006; Patel *et al.*, 2007; Caixeta *et al.*, 2009; Romar *et al.*, 2011). It is of general consensus that oocytes that do not complete the process of mRNAs synthesis until ovulation or OPU have poorer developmental competence (Fair *et al.*, 1995). In cattle, oocytes from COCs morphologically classified as grade 1 quality have greater amounts of mRNAs than poorer quality oocytes (Biase *et al.*, 2008). However, any correlation between mRNA amount and the developmental capacity to blastocyst was observed when the same oocyte was used for both the analysis of the quantity of transcripts and the developmental rate (Biase *et al.*, 2009). The feasibility of such an experiment required the creation of a new retrospective model that allowed the individual analysis of development. For that, the transcripts were quantified from cytoplasmic biopsies of mature oocytes parthenogenetically activated and individually cultivated (Biase *et al.*, 2009). Although these findings indicate that the global amount of mRNAs is not related to the embryo competence of development, more than a hundred genes were already described as differentially expressed between competent and incompetent oocytes (Robert *et al.*, 2000; Fair *et al.*, 2004; Gutiérrez-Adán *et al.*, 2004; Mourot *et al.*, 2006; Patel *et al.*, 2007; Caixeta *et al.*, 2009; Romar *et al.*, 2011). Thus, this same retrospective model described above was used to analyze differentially expressed genes between oocytes that have developed into blastocysts and those that were blocked at the 8-16 cells stage (Biase *et al.*, 2012). Twenty-nine genes were identified as differentially expressed, 16 of those have higher expression in the biopsies of the oocytes that developed into blastocysts, and 13 in the ones that were blocked at the 8-16 cells stage. A significant part of these genes were involved in the regulation of transcription, RNA processing and protein synthesis and degradation (Biase *et al.*, 2012). Moreover, other differentially expressed genes related to DNA repair and replication and also in the cellular cycle were detected (Biase *et al.*, 2012). Thus, these findings are in agreement with previous reports that indicate that the transcripts stored during the oocytes growth and maturation are important to determine their developmental competence (Robert *et al.*, 2000; Fair *et al.*, 2004; Gutiérrez-Adán *et al.*, 2004; Mourot *et al.*, 2006; Patel *et al.*, 2007; Caixeta *et al.*, 2009; Romar *et al.*,

*et al.*, 2011; Ripamonte *et al.*, 2012).

### Developmental competence and number of copies of mitochondrial DNA

During the oogenesis and folliculogenesis the number of copies of mitochondrial DNA (mtDNA) increases significantly, ending up with mature oocytes with hundreds of thousands of copies (Cao *et al.*, 2007; Shoubridge and Wai, 2007; Cree *et al.*, 2008; Wai *et al.*, 2008). The oocyte content of mtDNA is greater than what is found in any other cellular type (May-Panloup *et al.*, 2007), driving the attention to a potential importance of mitochondria for fertilization and early embryo development (Smith *et al.*, 2005; May-Panloup *et al.*, 2007; Shoubridge and Wai, 2007). Thus, oocytes with reduced number of copies of mtDNA may have poorer development or incapacity to develop after fertilization compared to oocytes with normal content of mtDNA. Despite their huge content, the number of copies of mtDNA considerably varies between different oocytes (Tamassia *et al.*, 2004; Smith *et al.*, 2005; May-Panloup *et al.*, 2007; Chiaratti *et al.*, 2010a). Although it remains unclear what determines such variation or its effects upon development (May-Panloup *et al.*, 2007), several studies have related the amount of mtDNA and fertility in many species (Reynier *et al.*, 2001; May-Panloup *et al.*, 2005a, 2007; El Shourbagy *et al.*, 2006; Santos *et al.*, 2006; Wai *et al.*, 2010). In humans, reduced amount of mtDNA was found in unfertilized oocytes with intrinsic abnormalities compared to oocytes that failed to fertilize because of impairments related to other factors (Reynier *et al.*, 2001). Although it seems evident that the depletion of the content of mtDNA is associated with oocyte disruption of competence (May-Panloup *et al.*, 2007), this relation is still controversial (Chiaratti and Meirelles, 2010; Chiaratti *et al.*, 2010a).

The same retrospective model described below was used to investigate the occurrence of a correlation between the number of copies of mtDNA and oocyte competence (Chiaratti *et al.*, 2010a). However, no differences were observed regarding the number of copies of mtDNA of oocytes that normally developed into blastocysts and the ones that blocked or lacked cleavage. Even the oocytes with 90% less mtDNA than average were capable of reaching the blastocyst phase (Chiaratti *et al.*, 2010a). These findings are contradicting with the general consensus that the amount of mtDNA affects oocyte competence to develop into blastocyst (Reynier *et al.*, 2001; May-Panloup *et al.*, 2005a, 2007; El Shourbagy *et al.*, 2006; Santos *et al.*, 2006; Wai *et al.*, 2010). A subsequent study was then designed to evaluate the developmental capacity of oocytes subjected to mitochondrial depletion (Chiaratti *et al.*, 2010a). Oocytes were centrifuged to concentrate the mitochondria in one extremity and allow the removal of the mitochondria-enriched cytoplasmic



fraction by aspiration. When the depleted oocytes were cultured they developed into blastocysts in similar rates of intact-control oocytes (Chiaratti *et al.*, 2010a), supporting our previous finding. Molecular analysis performed in the resulting embryos evidenced that the content of mtDNA is restored during its development to blastocyst when the oocyte is mitochondrial depleted (Chiaratti *et al.*, 2010a). The mtDNA reestablishment is accompanied by the overexpression of genes enrolled in the control of mtDNA replication (Chiaratti *et al.*, 2010a). This result suggests that when the oocytes have reduced number of copies of mtDNA (i.e. oocytes with 90% less mtDNA and capable to develop into blastocyst), the embryo is able to regulate the replication of the mitochondrial genome to attempt the energetic demands of the pre-implantation period (Thompson, 2000; Houghton and Leese, 2004; Dumollard *et al.*, 2007; May-Panloup *et al.*, 2007). This finding is opposed to previous studies that point out a relationship between mtDNA content and oocyte competence (Reynier *et al.*, 2001; May-Panloup *et al.*, 2005a, 2007; El Shourbagy *et al.*, 2006; Santos *et al.*, 2006; Wai *et al.*, 2010). The contradiction of results may lay in different causes. First, the level of mtDNA in mice is kept constant during the pre-implantation period (Smith *et al.*, 2005; Thundathil *et al.*, 2005; Cao *et al.*, 2007; Cree *et al.*, 2008; Wai *et al.*, 2008), differently from what is found to occur in cattle (May-Panloup *et al.*, 2005b; Smith *et al.*, 2005). This can explain the connection found between the oocyte competence and the content of mtDNA in mice (Wai *et al.*, 2010). In humans, it is possible that the reduced number of copies of mtDNA observed in unfertilized ova is consequence of a disruption in the machinery of mtDNA replication (Reynier *et al.*, 2001; May-Panloup *et al.*, 2005a; Santos *et al.*, 2006). On the other hand, in cattle, the oocytes subjected to the depletion of a cytoplasmic fraction concentrated with mitochondria were probably free of abnormalities and consequently able to restore their original stocks of mtDNA by activating the machinery of replication. Thus, the embryonic development was unaffected by initial depletion (Chiaratti *et al.*, 2010a). Abnormalities in the machinery of mtDNA replication were already reported in humans (Luoma *et al.*, 2004; Pagnamenta *et al.*, 2006). One example is the alteration in the expression of *TFAM*, a key gene that regulates mtDNA replication (Smith *et al.*, 2005; May-Panloup *et al.*, 2007).

#### **Cytoplasmic transfer as a tool to restore oocyte competence**

The cytoplasmic transfer was used in the late nineties to improve the results of assisted reproduction in women with recurrent fails in embryonic implantation after ICSI (intracytoplasmic sperm injection) or IVF (Cohen *et al.*, 1997, 1998; Huang *et al.*, 1999; Lanzendorf *et al.*, 1999). By this procedure, 5

to 15% of the cytoplasm from a presumed competent oocyte was transferred during the ICSI to the oocyte of a patient with fertility problems. This technique allowed the reestablishment of oocyte capacity to develop into a viable embryo and culminated in the delivery of healthy babies (Cohen *et al.*, 1997, 1998; Huang *et al.*, 1999; Lanzendorf *et al.*, 1999). Thus, it has been suggested that one or more cytoplasmic factors transferred during this procedure were responsible for rescuing development by sustaining the necessities of the incompetent oocyte (Barritt *et al.*, 2001; Chiaratti *et al.*, 2011b; Levy *et al.*, 2004; Poulton *et al.*, 2010). In domestic animals, the cytoplasm transfer has been more frequently used as a model to study mitochondrial inheritance (Steinborn *et al.*, 1998; Levy *et al.*, 2004; Chiaratti *et al.*, 2010b; Ferreira *et al.*, 2010; Sansinena *et al.*, 2011). However, considering the importance of cytoplasmic inheritance to the early embryonic development (Picton *et al.*, 1998; Meirelles *et al.*, 2004; May-Panloup *et al.*, 2007; Shoubridge and Wai, 2007), it can be potentially used to improve the fertility of animals with oocytes of reduced fertility. This may be applied, for example, to restore oocyte competence of repeat breeders Holstein cows. Recently, the low fertility of repeat breeder cows exposed to heat stress was attributed to oocyte disruption, with indicatives of cytoplasmic alterations (Ferreira *et al.*, 2011). If results are confirmed, the competence of development of oocytes recovered from repeat breeder cows can be potentially restored by the supplementation with a fraction of cytoplasm from oocytes of categories known as more fertile (i.e. Holstein heifers).

Based on the fore discussed data, several cytoplasmic factors can be responsible for developmental failures of incompetent oocytes (Picton *et al.*, 1998; Robert *et al.*, 2000; Meirelles *et al.*, 2004; May-Panloup *et al.*, 2007; Biase *et al.*, 2008, 2009, 2012; Chiaratti and Meirelles, 2010). Among these, the importance of the number of mtDNA copies per oocyte has been most discussed once it probably represented a limiting factor for its competence when cytoplasmic transfer was successfully used in humans (Barritt *et al.*, 2001; Levy *et al.*, 2004; Chiaratti *et al.*, 2011b). It is reasonable to consider that cytoplasm transfer introduces mitochondria in the recipient oocyte, what may restore its developmental competence by enhancing the content of mtDNA when this is limiting (Barritt *et al.*, 2001; Levy *et al.*, 2004; Chiaratti *et al.*, 2011b). However, due to previous discussed data rejecting the relation between mtDNA content and oocyte competence (Chiaratti and Meirelles, 2010; Chiaratti *et al.*, 2010a), we believe that this hypothesis is strongly questionable for cattle. Aiming to investigate this hypothesis, bovine oocytes were incubated with etidium bromate (EtBr) during the IVM (Chiaratti *et al.*, 2011a). The EtBr is known for its capacity of interfering on mtDNA replication, resulting in depleted copies in somatic cells (Chiaratti and Meirelles, 2006). When



oocytes treated with EtBr were fertilized, a decrease on blastocyst development was observed. This decrease was completely reversed by cytoplasmic transfer from oocytes that were not exposed to EtBr (Chiaratti *et al.*, 2011a). Hence, when the embryos that had their development capacity restored by cytoplasmic transfer were transferred to synchronized recipient cows, healthy calves were born (Chiaratti *et al.*, 2011a). Unexpectedly, neither the number of copies of mtDNA nor the mitochondrial function estimated by the analysis of the mitochondrial membrane potential nor the total amount of ATP were altered in EtBr-treated oocytes (Chiaratti *et al.*, 2011a). Because the treatment with EtBr can have affected another cytoplasmic components (i.e. RNAs, proteins, energetic substrates), the effect of EtBr on development may not be resulted by mtDNA replication disruption (Malter, 2011). Anyway, these results clearly demonstrated that cytoplasmic transfer can successfully restore the competence of compromised oocytes (Malter, 2011). Thus, the potential use of cytoplasmic transfer to improve the competence of oocytes to develop in viable blastocysts was reaffirmed. This technique can be an interesting strategy to restore fertility of females with low embryos yield.

### Perspectives on *in vitro* embryo production

#### *Increasing nuclear transfer embryo production and generation of gametes in vitro*

Reproductive biotechnologies have for long shown its use for the production, selection, and multiplication of valuable animals.

Cloning through nuclear transfer, indeed, is one of the biotechnologies discussed since the birth of the ewe Dolly, the first mammal derived from a somatic cell nuclear transfer (SCNT; Wilmut *et al.*, 1997). After Dolly, several other animal species were already produced in laboratories throughout the world.

SCNT provides the possibility of the oocyte cytoplasm to reprogram an already differentiated cell into a pluripotent status similar to the embryonic one, and then to generate a new organism. The factors present in the ooplasm, as well as their exact mechanism of action during the reprogramming of the differentiated nucleus are not yet fully known.

Particularly on farm animal production, the possibility of generating identical individuals is highly desirable when two main concerns are considered: the first, which has been employed since SCNT generation until now, is the production of high quality herds and animals carrying special characteristics. Such selected animals would be multiplied by large-scale cloning, evaluated, distributed to breeders or owners, and used in specific reproductive systems.

However, the second concern, which is probably the most striking opportunity enabled by

cloning regarding animal production, has not yet been accomplished. The large-scale production of identical commercial herds in a defined breeding system, similar to other livestock presenting homogeneous genetic lineages, and its derived products commercialized to specialized food or pharmaceutical companies, is still not viable nowadays due to the low efficiency of SCNT.

Although SCNT reprograms a differentiated nucleus, it remains an inefficient technique. Less than 5% of produced embryos generate healthy adult animals (Wilmut, 2002; Cibelli, 2007). Several studies have demonstrated nuclear reprogramming deficiencies in cloned embryos (Bourc'his *et al.*, 2001; Dean *et al.*, 2001; Rideout *et al.*, 2001; Santos *et al.*, 2003) leading to problems such as placental dysfunctions, large offspring syndrome, and hepatic and respiratory complications (Hill *et al.*, 1999; Heyman *et al.*, 2002; Meirelles *et al.*, 2010).

As mentioned before, the factors that determine the ability of the oocyte cytoplasm to reprogram the somatic cell nucleus have been under investigation. It is reported that cloning efficiency is inversely correlated to the differentiated status of the donor cell, suggesting that an undifferentiated nucleus is more likely to be remodeled and reprogrammed (Green *et al.*, 2007). Hence, cloning using embryonic stem cells (ESC; Rideout *et al.*, 2000; Humpherys *et al.*, 2001) have resulted in higher efficiency (Hiragi and Solter, 2005) when compared to the use of more differentiated cells such as lymphocytes (Inoue *et al.*, 2005) and fibroblasts (Wakayama *et al.*, 1999; Humpherys *et al.*, 2001; Wakayama and Yanagimachi, 2001; Hochedlinger and Jaenisch, 2002; Gong *et al.*, 2004; Blelloch *et al.*, 2006). Therefore, the selection of cell populations that are amenable to reprogramming, for example, the use of ESC as donor nuclei, may be important to increase the cloning efficiency (Solter, 2000).

Interestingly, embryonic stem cells derived from other species other than mouse and humans, for example farm animals, fail to maintain the pluripotent characteristics *in vitro*. Despite innumerable studies, they still lack consistency of pluripotency markers and have not produced chimeras. These cells, are, therefore named stem cells-like, are not able to maintain its characteristics during *in vitro* culture, hampering its use for cloning.

The use of truly pluripotent cells which can be maintained *in vitro* for long periods without losing pluripotency properties as nuclei donors on animal cloning, however, was enabled by the advent of genetic induction of differentiated cells into pluripotency after forced expression of pluripotency-related transcription factors (*OCT3/4*, *SOX2*, *KLF4*, and *C-MYC*, represented by OSKM; Takahashi and Yamanaka, 2006, Takahashi *et al.*, 2007). These cells, called induced pluripotent stem cells (iPS cells or iPSC) are actually a groundbreaking advent for stem cell research in farm animals, once controversially to ESC, iPSC can be



generated and *in vitro* cultured in these species, and moreover, they apparently show pluripotency patterns similar to those from human and murine ES cells.

Recent data from our lab indicate that bovine fetal fibroblasts can be *in vitro* reprogrammed into pluripotent after lentiviral transduction of murine OKSM (unpublished data). These cells were positive for several markers of pluripotency, therefore being characterized in a pluripotent status not yet reported in bovine ESC-like (Fig. 1).

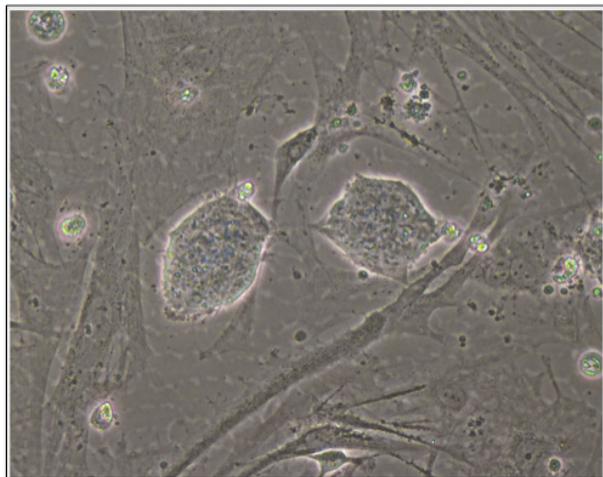


Figure 1. Bovine iPSC. 200X.

Recently the production of cloned swine and mice embryos after nuclear transfer of iPSC as nuclei donors was reported (Cheng *et al.*, 2012; Liu *et al.*, 2012). In our conditions, when bovine IPS (biPS) cells were used as nuclei donors, they were able to derive pre-implantation embryos. When these embryos were transferred to recipient cows, at least initial pregnancies could be established. However, cell cycle synchronization between biPS and oocyte needs to be optimized in order to allow a real comparison between developmental rates of embryos produced *in vitro*.

#### *The ability of in vitro gamete generation*

Another remarkable evidence that *in vitro* embryo production may benefit from nuclear reprogramming processes is the possibility of *in vitro* generation of functional gametes. Epigenetic studies conducted mainly based on cloned and induced reprogramming models have helped the ability of iPSCs in producing functional gametes, which may be helpful not only for the purpose of autologous treatment of several animal or human infertilities, but also, may avoid developmental problems found in SCNT-derived embryos.

When properly cultivated and maintained ES or iPSC cells have been shown to be able to generate structures similar to primordial germ cells (PGCs; Hubner *et al.*, 2003; West *et al.*, 2006). The induced

PGCs were able to develop into structures similar to oogonia, which are able to undergo meiosis, to recruit adjacent cells to form follicles and mediate the development to blastocyst after spontaneous parthenogenesis (Hubner *et al.*, 2003; Dyce and Li, 2006).

Female or male gamete-like structures have been derived *in vitro* in humans, mice and swine (Nagano, 2007). Recently, the generation of viable animals after induction of ES or iPSC cells into PGCs-like cells *in vitro* was reported. These cells were able to develop into gametes *in vivo*, which were recovered, submitted to IVM and IVF in mice (Hayashi *et al.*, 2012).

Cellular reprogramming in germinal cells is still a rare event, and similarities between such process and natural reprogramming need further studies. *In vitro* generation of functional gametes derived from other cell types from selected animals, without presenting the complications due to SCNT, may lead to a huge improvement on reproductive biotechnologies related to *in vitro* embryo production in a near future.

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## Endocrine conceptus signaling in ruminants

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### Abstract

The corpus luteum (CL) releases progesterone, which acts on the endometrium to induce release of histotroph that supports the free-floating conceptus and prepares for epithelial-chorial placentation. Two steroidogenic cell types, which are classified based on size, contribute to serum progesterone concentrations. Large luteal cells produce the bulk of progesterone because of constitutively active protein kinase A. Small luteal cells also contribute to serum progesterone concentrations through release of progesterone in response to luteotrophic stimuli. The CL is maintained in ruminants until endometrial-derived prostaglandin F2 alpha (PGF) initiates functional and structural regression. The decline in serum progesterone and loss of negative feedback on the hypothalamus and anterior pituitary sets up hormonal responses resulting in a new estrous cycle that is characterized by estrus, ovulation and formation of a new CL. If a conceptus is present, interferon tau (IFNT) is released from the conceptus, which binds receptors in the endometrium and prevents up-regulation of estrogen receptor (ESR1) and consequently oxytocin (OXT) receptor (OXTR). As a consequence, pulses of PGF are disrupted which results in rescue of the CL from luteolysis. In addition to these paracrine actions, early pregnancy also has direct endocrine action on the CL through inducing IFN-stimulated genes (ISGs) in the CL and resistance of the CL to PGF. Endocrine actions of IFNT have been described through detection of IFNT in uterine vein blood, induction of several ISGs in the CL during pregnancy, and following both *in vivo* (via miniosmotic pumps) and *in vitro* (in cultured small, large, and mixed luteal cells) delivery of recombinant ovine (ro) IFNT. These endocrine actions of IFNT might be applied to reducing embryo mortality and associated economic consequences in ruminants.

**Keywords:** corpus luteum, interferon-tau, luteolysis, pregnancy, prostaglandin F2 alpha.

### Introduction

This review provides a brief overview of the ovine CL in context of general steroidogenic and luteolytic mechanisms, which serves as a prelude to more detailed discussion of endocrine action of pregnancy on the CL. It provides some background on anti-luteolytic paracrine actions of conceptus-derived IFNT on the endometrium, but primarily focuses on more recent studies describing an endocrine role for pregnancy through induction of ISGs in the CL, as well as peripheral blood cell mononuclear cells (PBMC). Described herein is evidence to suggest that IFNT is released into the uterine vein in biologically relevant concentrations and has direct endocrine action on the CL resulting in resistance to the luteolytic actions of PGF. The induction of ISGs in the CL in response to pregnancy is described for selected targets such as ISG15, oligoadenylate synthetase (OAS), and myxovirus (influenza virus) resistance proteins (MX1 and MX2), but also more globally in context of preliminary studies that are introduced herein using microarray approaches. An infusion model using miniosmotic pumps to deliver recombinant ovine (ro) IFNT into the uterine vein is described in context of induction of ISGs in the CL and protection of the CL from both endogenous (Bott *et al.*, 2010) and exogenous (Bott *et al.*, 2010; Antoniazzi *et al.*, 2013) challenge with PGF. The objectives of the review are to provide an overview of recent studies supporting the concept that endocrine signaling occurs through release of conceptus-derived IFNT and actions of this cytokine in inducing ISGs and resistance of the CL to lytic effects of PGF.

### Luteal cells and luteolysis

Small and large luteal cells contribute to production of progesterone in the ovine CL (Fig. 1). Small luteal cells are more abundant than large luteal cells and are characterized by irregular shape, tapering cytoplasmic processes, predominantly smooth endoplasmic reticulum, mitochondria with tubular and lamellar

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cristae, and one or more Golgi complexes (O'Shea *et al.*, 1979). Large luteal cells synthesize and produce more progesterone, and are less responsive to luteinizing hormone (LH) than small luteal cells (Rodgers and O'Shea, 1982). Both large and small luteal cells have LH receptors, however large luteal cells release progesterone independently of LH action because of constitutively active protein kinase A and increased phosphorylation of steroidogenic acute regulatory protein (reviewed in Niswender *et al.*, 2007).

PGF is recognized as the luteolytic hormone in sheep (McCracken *et al.*, 1972). Continued exposure of endometrium to progesterone during the late luteal phase causes down-regulation of progesterone receptor and, consequently, up-regulation of ESR1, OXT, OXTR, and pulsatile release of PGF (reviewed in Spencer *et al.*, 2007). During the early luteolytic phase (days 12-14), exposure of large luteal cells to limited pulses of PGF activates protein kinase C (PKC) and release of OXT (Wiltbank *et al.*, 1989). PGF inhibits steroidogenesis, but also induces cyclooxygenase (COX)-2 (Silvia *et al.*, 1984) which may increase the concentration of intraluteal PGF in large luteal cells. Intraluteal progesterone may prevent the actions of OXT on small luteal cells and ability of PGF to increase

calcium in large luteal cells (Davis *et al.*, 1992). Later in the luteolytic process, by day 16, when release of progesterone has diminished by 80% and OXT binds to receptors on small luteal cells, secretion of progesterone is inhibited and intracellular levels of calcium increase which leads to apoptosis. Secretion of PGF from large luteal cells may be facilitated through a transporter (SLCO2A1) and through autocrine action on large luteal cells to further stimulate OXT release, PKC, and COX-2 activity. Intraluteal PGF acts on large cells through increasing intracellular calcium, which likely leads to apoptosis of these cells. PGF also activates PKC mediated increases in early growth response 1 (EGR1) and transforming growth factor  $\beta$  (TGFB1) during bovine luteal regression *in vivo* and *in vitro* (Hou *et al.*, 2008). Repression of insulin-like growth factor (IGF-1) and cell-survival responses by PGF have been demonstrated in *in vivo* and *in vitro* bovine CL models (Arvais *et al.*, 2010). These luteolytic responses in large and small luteal cells are the focus of possible disruption through endocrine actions of pregnancy and more specifically IFNT. For example, cell-survival responses might be maintained in the CL during early pregnancy in response to IFNT, which counter apoptotic responses induced by PGF and are the focus of our present studies.

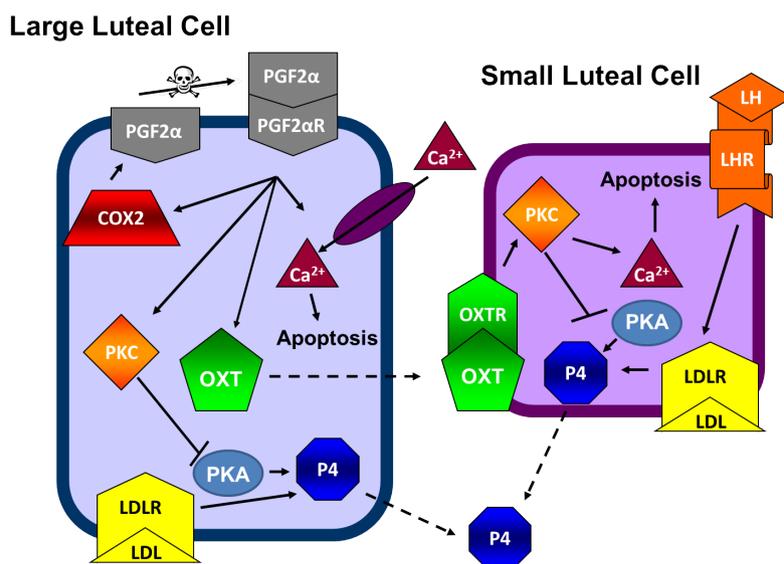


Figure 1. Small and large luteal cell responses during luteolysis (day 16) in ewes. See text above for detail. Diagram is adapted from Niswender *et al.* (2007), with permission. Abbreviations not defined in text are: low density lipoprotein (LDL) receptor (LDLR), progesterone (P4), protein kinase A (PKA).

### Establishment of early pregnancy in sheep

Moor and Rowson (Moor and Rowson, 1966b) and Mapletoft and co-workers (Mapletoft *et al.*, 1976b) described local/ipsilateral effect of the conceptus in maintaining the CL during early pregnancy. These studies also were interpreted to mean that there is no systemic mediator of rescue of the CL, because ligation of the gravid horn protected the CL ipsilateral to the

conceptus, while the contralateral CL regressed. However, these studies do not discount possible endocrine action of the conceptus on the CL in context of resistance to PGF and longer-term survival of the CL during early pregnancy. For example, several investigators have described the CL of pregnancy to be more resistant to lytic effects of PGF (Inskeep *et al.*, 1975; Mapletoft *et al.*, 1976a; Pratt *et al.*, 1977; Silvia and Niswender, 1984). Exactly why and how this



resistance to PGF occurs in the CL during pregnancy is unknown.

Pregnant and cyclic ewes have very different patterns of PGF release as reflected by PGF metabolite (PGFM) in the blood 12-16 days post ovulation (Thorburn *et al.*, 1972; Zarco *et al.*, 1988a). Peak production of PGF occurs at day 14-15, regardless of pregnancy status. Estrous cycling ewes release PGF in a pulsatile manner, while pregnant ewes have a pattern of more constant and slower increases in the release (Peterson *et al.*, 1976; Zarco *et al.*, 1988b). More PGF is found exiting the uterus through the uterine vein in day 13 pregnant vs estrous cycling ewes (Wilson *et al.*, 1972). The antiluteolytic antagonism of PGF release from the endometrium during pregnancy is regulated by conceptus-derived IFNT, which contributes to rescue of the CL. However, the release of PGF from the endometrium is not ablated completely and there is a possibility that the CL produces PGF (Silva *et al.*, 2000). Thus, mechanisms inducing resistance of the CL to PGF may need to be activated during early pregnancy in the ewe to prevent luteolysis. We propose herein that IFNT is released into the uterine vein and actually has direct endocrine actions on the CL to induce putative cell survival, anti-apoptotic and, consequently, antiluteolytic responses.

The luteolytic effect of the non-pregnant uterus can be blocked through transfer of ovine embryos into the uterus by day 12 (Moor and Rowson, 1966a, b). For this reason, day 10-12 ovine conceptuses were examined, which resulted in identification of IFNT (also called trophoblast proteins or protein X; Imakawa *et al.*, 1987, 1989) as the primary conceptus secretory protein (Godkin *et al.*, 1982) responsible for altering endometrial release of PGF (Zarco *et al.*, 1988a) and rescuing the CL during pregnancy in sheep. IFNT acts through silencing endometrial transcription of ESR1 and, consequently, OXTR, thereby disrupting pulsatile release of PGF in response to OXT (Spencer *et al.*, 1995b; Spencer and Bazer, 1996). Thus, IFNT acts through paracrine anti-luteolytic action on the endometrium to protect the CL during maternal recognition of pregnancy.

IFNT has been shown to stimulate Janus kinase signal transducers and activators of transcription (STATs; Binelli *et al.*, 2001; Thatcher *et al.*, 2001), IFN regulatory factors (IRFs; Perry *et al.*, 1999) and to modulate COX-2 and phospholipase A2 (Binelli *et al.*, 2000) in bovine endometrium. Also, pregnancy and more specifically IFNT induces many ISGs in the endometrium and in blood cells (for review see Hansen *et al.*, 2010a). One example of a pregnancy-associated ISG that is expressed both in the endometrium (Austin *et al.*, 1996; Johnson *et al.*, 1999b) and blood cells (Han *et al.*, 2006; Hansen *et al.*, 2010a) is ISG15, an ubiquitin homolog that resembles a tandem ubiquitin repeat which ends in carboxyl terminal LRLRGG. This C-terminal feature allows these post-translational

modifiers and regulatory proteins to become covalently attached to proteins via a Gly:Lys isopeptide bond that ligates ubiquitin (Wilkinson and Audhya, 1981; Ecker *et al.*, 1987) and ISG15 (Loeb and Haas, 1992; Narasimhan *et al.*, 1996) to targeted proteins (reviewed in Haas, 2007). Polyubiquitinated proteins are thought to be targeted for degradation through the 26S proteasome, whereas ISGylated proteins might be stabilized or altered for specialized function such as RNA splicing, chromatin remodeling/polymerase II transcription, cytoskeletal organization and regulation, stress responses, translation and viral replication (Malakhova *et al.*, 2003; Giannakopoulos *et al.*, 2005; Zhao *et al.*, 2005; Takeuchi *et al.*, 2006). ISG15 serves as an excellent marker for IFNT action and is a conserved uterine response to pregnancy in primates (Bebington *et al.*, 1999a, b; Bebington *et al.*, 2000), mice (Austin *et al.*, 2003; Bany and Cross, 2006), and cattle (Austin *et al.*, 1996; Hansen *et al.*, 1997; Johnson *et al.*, 1998; Perry *et al.*, 1999; Thatcher *et al.*, 2001).

### Pregnancy induces ISGs in peripheral tissues

Originally, IFNT was not thought to be released from the uterus and was believed to have only paracrine action on the endometrium because it was not detected in peripheral blood. Because ISG15 was localized to endometrial tissues subjacent to the luminal epithelium, including the myometrium (Johnson *et al.*, 1999a, b), the presence of surrogate mediators of IFNT, that induce these ISG responses, were suspected. Likewise, ISG mRNAs were found to be up-regulated in PBMC in response to pregnancy in both sheep (Yankey *et al.*, 2001) and cattle (Han *et al.*, 2006; Gifford *et al.*, 2007). The impact of pregnancy on induction of ISGs in blood cells was intriguing, especially in light of opinions that the conceptus acted locally in paracrine manner as an anti-luteolysin. Exactly how PBMC became activated to express ISGs was unknown. However, at least 674 genes were up-regulated and 721 genes were down-regulated in blood cells in response to pregnancy on day 18 in cattle (Hansen *et al.*, 2010a). Many of these genes were ISGs. In search of a mechanism to describe release of IFNT from the uterus and explain up-regulated ISGs in PBMC, we examined lymph nodes draining the uterus (iliac) and the head (submandibular) from day 15 pregnant ewes and found no difference in ISGs expression, suggesting that IFNT was not released into the uterine lymphatics and this was not a pathway through which IFNT induced ISGs in PBMC.

Uterine vein blood from pregnant or non-pregnant sheep was examined for antiviral activity in order to evaluate the possibility that IFNT was released systemically from the uterus. Surprisingly, significant antiviral activity was found in uterine vein blood from day 15 pregnant sheep. IFN released from the uterus was in the amount of  $\sim 200 \mu\text{g}$  ( $2 \times 10^7 \text{ U}$ )/24 h when converted based on bioactivity of IFN standards



(Oliveira *et al.*, 2008). The uterine venous blood had 500- to 1000-fold higher concentrations of bioactive IFN than uterine arterial blood on day 15 of pregnancy. To determine if this antiviral activity was caused by IFNT or some other type I IFN, uterine vein blood was preadsorbed with antibody against IFNT and then tested in the antiviral assay against blood that was preadsorbed with non immune normal rabbit serum (Bott *et al.*, 2010; Fig. 2).

Preadsorption of uterine vein blood with normal rabbit serum had no effect on the high antiviral activity detected on day 15 of pregnancy. In contrast,

preadsorption of uterine vein blood with antiserum against IFNT completely blocked antiviral activity found on day 15 of pregnancy. It was concluded that the major contributor to antiviral activity in uterine vein blood on day 15 of pregnancy was IFNT. This conclusion is further supported by detection of IFNT in uterine vein blood by mass spectroscopy (Romero and Hansen; Colorado State University, Fort Collins, CO; provisionally accepted in *Physiol Genomics*) and by using a specific and sensitive radioimmunoassay for IFNT (Antoniazzi and Hansen; Colorado State University, Fort Collins, CO; unpublished results).

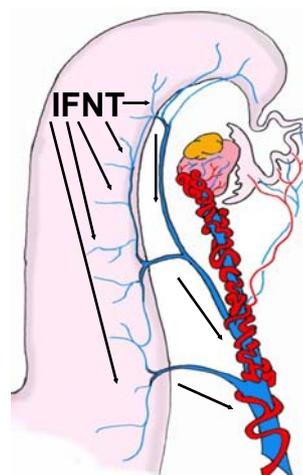
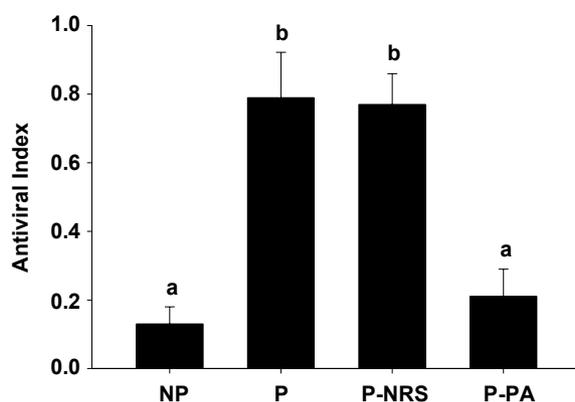


Figure 2. Preadsorption of uterine vein blood blocks antiviral activity detected in uterine vein blood from day 15 pregnant sheep. Left panel describes antiviral index and is adopted from Bott *et al.* (2010). NP: uterine vein blood from day 15 non pregnant sheep. P: uterine vein blood from day 15 pregnant sheep. P-NRS: uterine vein blood from day 15 pregnant sheep preadsorbed with normal rabbit serum. P-PA: Uterine vein blood from day 15 pregnant sheep preadsorbed with antiserum against IFNT. Based on these data it is concluded that the primary IFN released from the uterus into the uterine vein is IFNT (right panel).

### Induction of ISGs by IFNT and pregnancy in the CL

Pregnancy (the conceptus) has endocrine effects through inducing ISGs in extrauterine tissues such as the CL (Oliveira *et al.*, 2008; Bott *et al.*, 2010). For example, ISG15 mRNA concentrations were up-regulated in CL from day 15 pregnant compared to non-pregnant ewes. Likewise, ISG15 protein and its ISGylated protein targets also were up-regulated in CL in response to pregnancy. Immunohistochemical staining using an anti-ISG15 monoclonal antibody (clone 5F10; Austin *et al.*, 2004) revealed that ISG15 was predominantly localized to large luteal cells on day 15 of pregnancy, with diminished, but significant localization to small luteal cells. To confirm that these ISGs were pregnancy-associated and induced by IFNT, small, large, and mixed luteal cells which were isolated on day 10 of the estrous cycle and cultured with rIFNT for 24 h demonstrated significant induction of ISG15

(Antoniazzi *et al.*, 2013). Similar cultures of day 10 CL with prostaglandin E2 revealed no impact on production of ISG15. In order to more globally examine induction of ISGs in the CL by pregnancy, we collected CL on day 12 or 14 of pregnancy or the estrous cycle and isolated and screened mRNA using the bovine Affymetrix microarray. The specific days of pregnancy/estrous cycle were selected based on serum progesterone levels, which did not change in pregnant but started to decline from day 12 to 14 with a significant decline by day 15 of the estrous cycle (Fig. 3). There were 21 differentially expressed genes in CL from day 14 pregnant compared to day 12 non-pregnant ewes and 734 differentially expressed genes in CL from day 14 pregnant compared to day 14 non-pregnant CL (Romero and Hansen; Colorado State University, Fort Collins, CO, provisionally accepted in *Physiol Genomics*). Many of the genes differentially expressed in response to pregnancy were ISGs (type I IFN signaling).

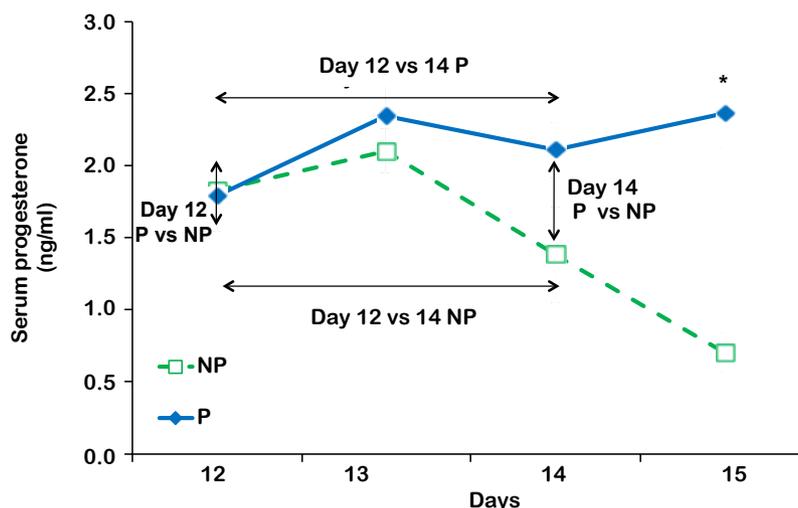


Figure 3. Serum progesterone and experimental design for microarray analysis of CL on days 12 and 14 of the estrous cycle and pregnancy. Microarray analysis revealed induction of many ISGs in response to pregnancy/IFNT. Data adapted from Romero *et al.* (provisionally accepted in *Physiol Genomics*). Note that day 0 in these studies is day of estrus.

### Systemic delivery of IFNT

Many groups have examined infusion of roIFNT into the uterine lumen as well as subcutaneous injections and other systemic delivery approaches (Vallet *et al.*, 1988; Miranda *et al.*, 1991; Spencer *et al.*, 1995a, 1999). A general overview of these studies is that a paracrine effect was noted in altering the ESR1/OXTR/PGF system with little reported impact directly on the CL (Vallet *et al.*, 1988; Martal *et al.*, 1990; Green *et al.*, 2005). These studies were interpreted to mean that IFNT acted in paracrine action to extend the luteal phase.

Systemic delivery (Nephew *et al.*, 1990; Martinod *et al.*, 1991; Schalue-Francis *et al.*, 1991; Davis *et al.*, 1992) of mg quantities of IFNT through intramuscular or subcutaneous injection also was studied to determine if it extended inter-estrous interval as well as ability to increase fertility. In some cases the amounts of IFNT used actually induced hyperthermia, and had no effect or caused a decline in fertility (Niswender *et al.*, 1997; Ott *et al.*, 1997). These doses of roIFNT have been reduced to  $2 \times 10^7$  U (200  $\mu$ g) in intrauterine deliveries to avoid hyperthermia and high death loss of ewes (Spencer *et al.*, 1999). Induction of ISG15 has been reported in the CL following subcutaneous (Spencer *et al.*, 1999) and intramuscular injections (Chen *et al.*, 2006) of roIFNT given between days 11-17. Chen *et al.* (2006) described inter-estrous interval of 32.7 days in ewes that received intrauterine infusions of 200  $\mu$ g roIFNT, but an average interval of only 17 and 22 days in ewes that were injected (i.m.) with 200  $\mu$ g or 2 mg roIFNT, respectively. The induction of ISG15 in the CL occurred in response to infusion and injection of 2 mg roIFNT, but not following injection of 200  $\mu$ g roIFNT. None of these systemic methods of roIFNT treatment induced a pseudopregnant state that continued for more than a few

days. However, both methods of roIFNT delivery were able to induce ISGs in the endometrium.

A new model was developed to deliver IFNT into the uterine vein by using surgically implanted miniosmotic pumps (Fig. 4). This was done to allow for systemic delivery of IFNT but also to provide an opportunity for the IFNT to have access to the utero-ovarian plexus in the event that it happened to cross over to the ovarian artery from the uterine vein. Delivery of 200  $\mu$ g over 24 h was selected as an appropriate amount based on estimate of antiviral activity and relevant concentration of IFNT determined through this bioassay (see Hansen *et al.*, 2010a for more detailed rationale). Considering blood volume in sheep it is estimated that systemic levels in circulation will stabilize around 2.4 ng/ml.

After infusing 200  $\mu$ g IFNT into the uterine vein per day, it was clear that this was enough to cause induction of ISG15 mRNA in ipsilateral and contralateral CL as well as in endometrium and liver (Oliveira *et al.*, 2008; Bott *et al.*, 2010). This systemic delivery, when continued from day 10 to day 17 of the estrous cycle, caused a delay in the return to estrus in all ewes with normal serum progesterone profiles prior to insertion of the miniosmotic pumps on day 10. All control (bovine serum albumin; BSA)-infused ewes returned to estrus by day 19, whereas all IFNT infused ewes had a delay in return to estrus that may have been extended beyond day 32, which is when this study was terminated and necropsy was performed to confirm presence of the original CL. It was concluded from these studies that endocrine delivery of IFNT into the uterine vein was able to block luteolysis from endogenously produced PGF if delivery occurred from days 10 to 17 of the estrous cycle. However, these studies did not directly test the ability of IFNT to protect the CL from lytic actions of PGF.

The first study to use miniosmotic pump delivery to investigate this was completed by Bott *et al.*



(2010) where 200  $\mu\text{g}$  roIFNT was delivered into the uterine vein starting on day 10 (prior to endogenous action of PGF) for 12 h, at which time a single injection of PGF (Lutalyse; 4 mg/58 kg i.m.) (Silva and Niswender, 1984, 1986; Silva *et al.*, 2000; Bott *et al.*, 2010) was administered and exposure to roIFNT continued for another 12 h at which time ewes were necropsied. Serum progesterone concentrations declined significantly in BSA-infused ewes following injection

with PGF. In roIFNT-infused ewes, serum progesterone declined slightly following the PGF injection but to levels that were not different from BSA-infused controls. More recently we modified this experiment and demonstrated that infusion of only 20  $\mu\text{g}/\text{day}$  into the uterine vein for three days from day 10 to 13 was able to significantly protect the CL from lytic action of PGF exogenously administered on day 11 (Fig. 5; Antoniazzi *et al.*, 2013).

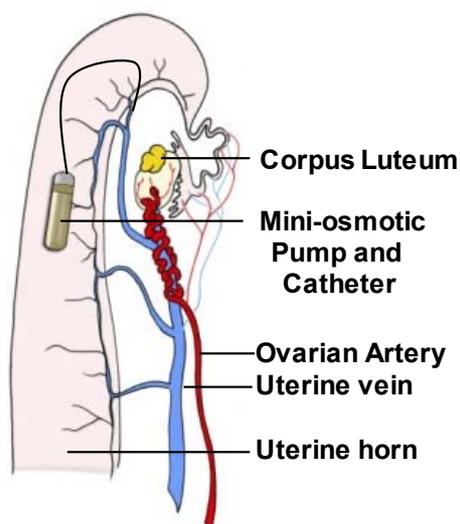


Figure 4. Description of miniosmotic pumps and catheterization of the uterine vein upstream of the utero-ovarian plexus for endocrine delivery of roIFNT. See Oliveira *et al.* (2008); Bott *et al.* (2010); Hansen *et al.* (2010a); Antoniazzi *et al.* (2013). Adapted from Bott *et al.* (2010).

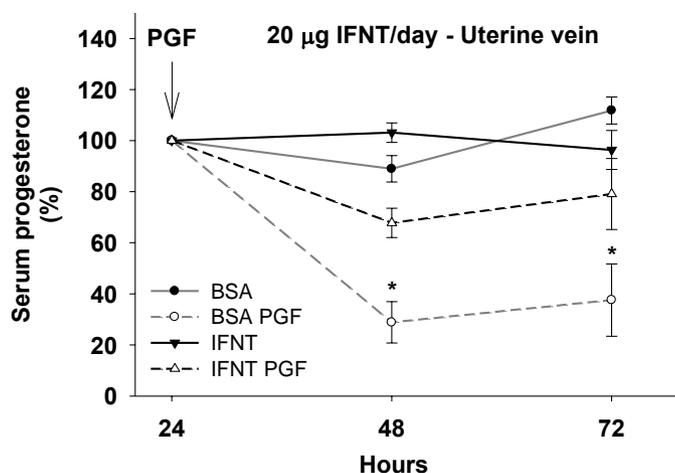


Figure 5. Serum progesterone concentrations following three day (10-13) infusion of BSA, BSA with PGF injection on day 11, IFNT or IFNT with PGF injection on day 11. Data were normalized to serum progesterone levels in ewes on day 11 (i.e., 24 h). Note that infusion of 20  $\mu\text{g}$  roIFNT/day into the uterine vein protected the CL from lytic action of exogenously administered PGF. Adapted from Antoniazzi *et al.* (2013).

### Conclusions

The ovine conceptus releases IFNT into the uterine lumen, where it has paracrine action on luminal epithelium to modify pulsatile release of PGF. In addition to well-studied action on the ESR1/OXTR/PGF axis, IFNT also induces many ISGs in the endometrium. These ISGs may be involved with preparing the uterine

endometrium for epitheliochorial placentation and formation of placentomes. However, they may also contribute to a peripheral resistance to infection through upregulation of innate immune responses. For example, many of the ISGs induced in endometrium and in blood cells are major cellular responders to viral infection. ISGs such as ISG15, IRFs, ubiquitin activating enzyme E1-like, MX2, retinoic acid-inducible gene 1, OAS are



upregulated in response to pregnancy in the endometrium and the blood and also are now described in the CL. Currently, it is not known why these ISGs are so massively and systemically upregulated in sheep. Perhaps ruminants have developed this provoked and upregulated innate response machine to help protect the fetus from maternal infection with virus, which in some cases can result in persistent infection in the fetus (Fig. 6; Hansen *et al.*, 2010a, b). The induction of ISGs in the CL may contribute to protection (resistance) of the CL to PGF.

The induction of ISGs in the CL in addition to several other pregnancy-associated genes may confer resistance in the CL to lytic action of PGF. Infusion of roIFNT into the uterine vein for seven days caused significant delay in return to estrus and may have not only had an impact on endometrial PGF release, but also

on the resistance of the CL to PGF. The later was directly tested through delivering IFNT into the uterine vein followed by an injection of exogenous PGF. Pre-treatment with IFNT for 12 h tended to block and with 24 h infusion of roIFNT blocked the decline in serum progesterone caused by injection of PGF. This direct action of IFNT in protecting the CL was replicated by using 10-fold lower concentration of roIFNT (20 instead of 200 µg/day) delivered into the uterine vein. Exactly how IFNT protects the CL from PGF is the focus of future experiments designed to examine signal transduction mechanisms coupled to type I IFN receptors, the role of ISGs, but also disruption of lytic-cell death responses to PGF. Through uncovering these relationships, it may become possible to apply this knowledge to improving embryo survival and fertility.

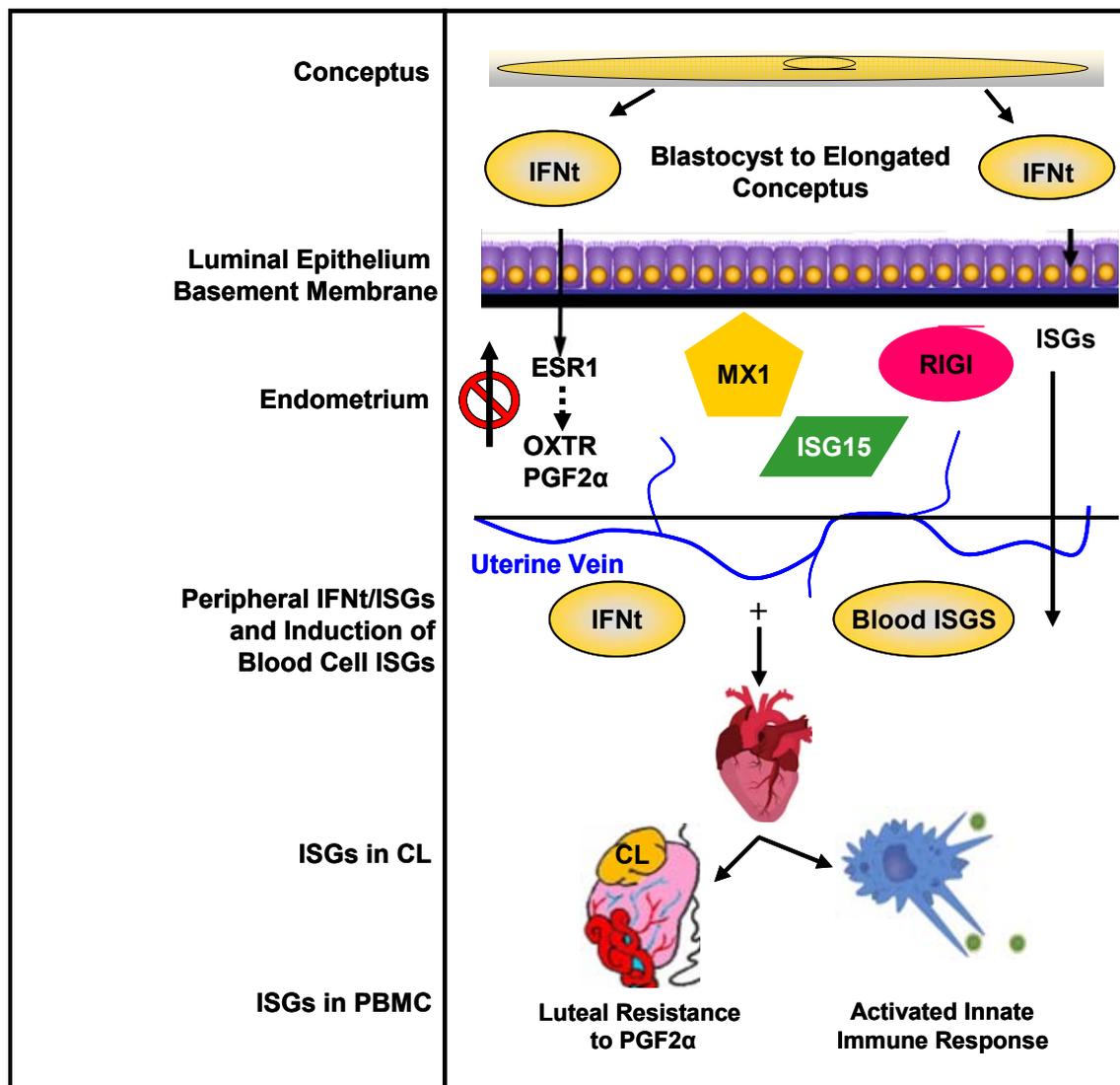


Figure 6. Paracrine and endocrine mechanism of IFNT action. IFNT is released from trophoblast of the expanding blastocyst, binds receptors, regulates the ESR1, OXTR and PGF axis and also induces massive ISGs response in the endometrium. IFNT also has endocrine action based on detection in uterine vein blood and induction of ISGs in peripheral tissues such as PBMC, liver and CL. Why these ISGs are induced in peripheral tissues like the CL is unknown. However, the ISGs do have very clear and documented roles in the innate immune response to viral expression.



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## Cellular and molecular basis of therapies to ameliorate effects of heat stress on embryonic development in cattle

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### Abstract

Much of the effect of heat stress on establishment and maintenance of pregnancy involves changes in ovarian function and embryonic development that reduce the competence of the oocyte to be fertilized and the resultant embryo to develop. There are three possible therapeutic approaches to manipulate the connection between hyperthermia and cellular responses to elevated temperature to improve fertility during heat stress. Embryo transfer is based on the idea that 1) most effects of heat stress on fertility involve actions during folliculogenesis or on cleavage-stage embryos and 2) the embryo has acquired resistance to elevated temperature by the time it is transferred at the morula or blastocyst stage. The mechanisms for acquisition of thermotolerance involve changes in production of reactive oxygen species in response to heat shock as well as accumulation of antioxidants in the embryo. Synthesis of heat shock proteins may not be the controlling factor for acquisition of thermotolerance because transcript abundance for *HSPA1A* and *HSP90AA1* is higher for the two-cell embryo than morula. Involvement of reactive oxygen species in actions of elevated temperature on embryo survival is indicative that provision of antioxidants to heat-stressed cows could improve fertility. More work is needed but there are indications that pregnancy rates can be improved by feeding supplemental  $\beta$ -carotene or administration of melatonin implants. It is also evident that there are genes that control thermotolerance at the cellular level. Brahman, Nelore and Romosinuano embryos have increased resistance to heat shock as compared to Holstein or Angus embryos. Mutations in the gene for heat shock protein 70 that control resistance of cells to heat shock have been identified in Holsteins. Selection for the desirable alleles of genes conferring cellular thermotolerance could lead to development of strains of cattle whose fertility is resistant to disruption by heat stress. Pursuing these and other therapeutic approaches for reducing consequences of heat stress for livestock species should be a priority because of the prospects for continuing global climate change.

**Keywords:** antioxidants, cattle, embryo transfer, genetics, heat stress.

### Introduction

Mammals can maintain a constant body temperature under a wide range of environmental

conditions but they do not function well when body temperature rises above the regulated temperature. This is especially the case for fertility (Hansen, 2009). Females of all mammalian species experience infertility when exposed to a heat stress of sufficient magnitude to cause hyperthermia, as has been demonstrated for cattle, mice, rabbits, sheep and pigs (Dutt, 1964; Tompkins *et al.*, 1967; Ulberg and Burfening, 1967; Ealy *et al.*, 1993; Matsuzuka *et al.*, 2005a, b). In dairy cattle, it has been estimated that conception rate begins to decline when uterine temperature rises about 0.5°C above normal (Gwazdauskas *et al.*, 1973). The magnitude of heat stress effects depend upon genetic and physiological adaptations that determine regulation of body temperature. Thus, fertility of *Bos indicus* x *B. taurus* females were less affected by heat stress than fertility of *B. taurus* females (Turner, 1982). Similarly, the increased heat production caused by milk synthesis makes lactating females less able to regulate body temperature (Cole and Hansen, 1993) and more sensitive to the anti-fertility effects of heat stress than non-lactating females (Badinga *et al.*, 1985).

Much of the effect of heat stress on establishment and maintenance of pregnancy involves changes in ovarian function and embryonic development that together reduce the competence of the oocyte to be fertilized and the resultant embryo to develop. It is possible to manipulate the connection between hyperthermia and cellular responses to elevated temperature to improve fertility during heat stress. Here will be discussed three therapeutic approaches for doing so and what is known regarding the cellular and molecular basis for their efficacy. One of these three approaches, embryo transfer, has been repeatedly demonstrated to greatly reduce the magnitude of infertility associated with maternal heat stress. The second approach, manipulation of the antioxidant status of the female, has not yet been reduced to practice and has yielded equivocal results in the field. The third approach, selection for genes controlling cellular thermotolerance, could lead to development of lines of cattle with superior genetic resistance to heat stress. The demonstration that there are genetic differences in embryonic resistance to elevated temperature (i.e, heat shock) means that such genes exist but their identity remains largely unknown.

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### Embryo transfer - Bypassing damage to the oocyte and cleavage-state embryo

Embryo transfer represents the only method currently available to improve fertility during heat stress that is based on manipulating physiology of the cow. The other methods involve cooling cows to reduce magnitude of heat stress (Flamenbaum and Galon, 2010). Large improvements in fertility during the summer can be achieved with embryo transfer (Putney *et al.*, 1989a; Ambrose *et al.*, 1999; Drost *et al.*, 1999; Al-Katanani *et al.*, 2002a; Rodrigues *et al.*, 2004; Block *et al.*, 2010; Stewart *et al.*, 2011; see Fig. 1 for examples). As shown in Fig. 1B, the summer decline in fertility can be largely eliminated. Embryo transfer can be expensive and the cost effectiveness of the procedure depends on maintaining a high pregnancy success using a low-cost embryo (De Vries *et al.*, 2011; Ribeiro *et al.*, 2012). One way to reduce the cost is to produce embryos *in vitro* using abattoir-derived oocytes. The promise represented by use of *in vitro* produced embryos has been limited by problems with vitrification (Ambrose *et al.*, 1999; Drost *et al.*, 1999; Al-Katanani *et al.*, 2002; Block *et al.*, 2010; Stewart *et al.*, 2011) and reduced competence of embryos to establish pregnancy as compared to embryos produced *in vivo* (Farin and Farin, 1995; Numabe *et al.*, 2000).

At the current level of embryo transfer technology, the improvement in pregnancy rates over artificial insemination is not a general feature of embryo transfer. In cases where heat stress was not present or cows were not inherently infertile (i.e., repeat-breeder cows; Son *et al.*, 2007; Block *et al.*, 2010; Canu *et al.*, 2010), there was no difference in pregnancy rate between inseminated cows and those receiving embryos (Sartori *et al.*, 2006; Rasmussen *et al.*, 2013). The lack of advantage for embryo transfer in the absence of heat stress is apparent by examination of Fig. 1B to view the differences between embryo transfer and artificial insemination in the winter.

Embryo transfer improves fertility during heat stress because it bypasses loss of pregnancies caused by damage to the oocyte and preimplantation embryo. To better illustrate the biological basis of the effectiveness of embryo transfer for improving fertility during heat stress, let us consider the timing of effects of heat stress on the oocyte and preimplantation embryo. It is the exploitation of this timing that makes ET effective at improving fertility during heat stress.

#### *Timing of heat stress effects on the oocyte*

Heat stress affects embryonic development long before the embryo is formed because it disrupts the process of oogenesis. This consequence of heat stress is indicated by observations that competence of oocytes to be fertilized and/or develop to the blastocyst stage is lower in summer than in winter. This is true both following insemination of cows *in vivo* (Sartori *et al.*, 2002) and after oocytes are recovered and subjected to *in*

*vitro* fertilization (Rocha *et al.*, 1998; Al-Katanani *et al.*, 2002b; Ferreira *et al.*, 2011) or chemical activation (Zeron *et al.*, 2001). Should fertilization succeed despite damage to the oocyte, the newly formed embryo remains susceptible to damage caused by heat stress. Exposure to heat stress *in vivo* (Putney *et al.*, 1988a; Ealy *et al.*, 1993) or elevated temperature *in vitro* (Edwards and Hansen, 1997; Sakatani *et al.*, 2004, 2012; Eberhardt *et al.*, 2009) reduces the competence of the cleavage-stage embryo to develop to the blastocyst stage.

The process of oogenesis is a long one - it takes about 16 weeks for a primordial follicle to grow to the point where it exerts dominance (Webb and Campbell, 2007) - and it is not clear how early in the process heat stress can disrupt oocyte development. In one experiment with Gir cows, a 28-day period of heat stress (achieved by placing cows in environmental chambers) resulted in reduced oocyte competence for *in vitro* fertilization as late as 105 days after the end of heat stress (Torres-Júnior *et al.*, 2008). It follows, therefore, that heat stress affects oogenesis at early stages of follicular growth. In another study, Roth *et al.* (2001b) found that exposure of lactating Holstein cows to heat stress of 12 h duration affected androstenedione production of cultured thecal tissue isolated from preovulatory follicles collected 28 days after heat stress.

Sensitivity of the early growing follicle to heat stress is a likely explanation for the fact that oocyte quality is only gradually restored in the autumn and that restoration of oocyte competence for cleavage in the autumn can be hastened by treatments that increase follicular turnover (Roth *et al.*, 2001a, 2002).

Disruption of oogenesis could be the result of actions of elevated temperature on the follicle or oocyte or to alterations in the endocrine control of folliculogenesis. Culture of cumulus cells and thecal cells at elevated temperature has sometimes been reported to reduce steroid secretion (Wolfenson *et al.*, 1997). Hormones involved in follicular growth and oocyte function whose concentrations in blood are changed by heat stress including luteinizing hormone (Wise *et al.*, 1988a; Gilad *et al.*, 1993), estradiol-17 $\beta$  (Wolfenson *et al.*, 1997), and progesterone (Wolfenson *et al.*, 2000). Concentrations of progesterone before ovulation can affect subsequent fertility (Bisinotto *et al.*, 2010; Denicol *et al.*, 2012), possibly because of actions on oocyte function, and a reduction in circulating progesterone concentrations before ovulation caused by heat stress (Wolfenson *et al.*, 2000) could conceivably compromise the oocyte.

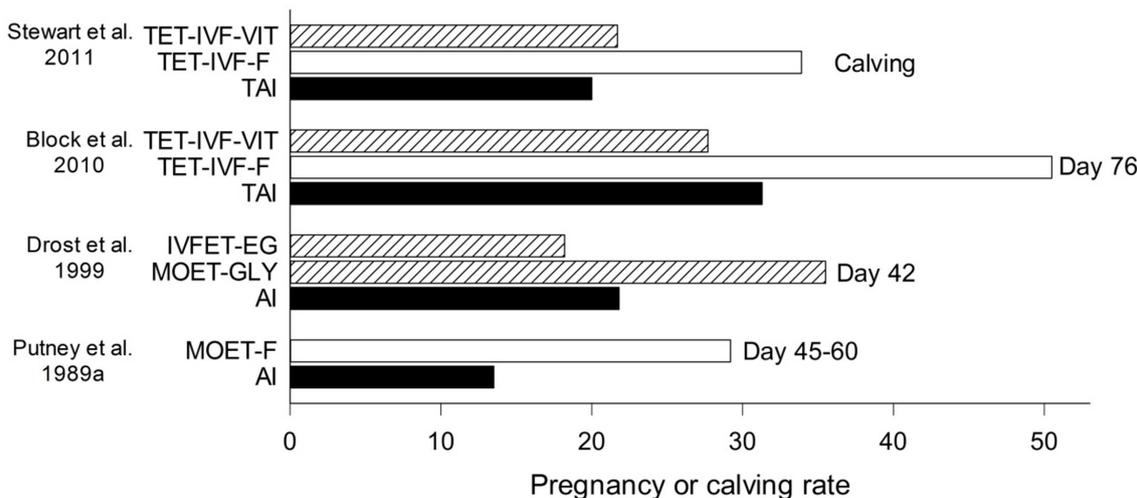
The oocyte remains sensitive to heat stress through the period of oocyte maturation. The proportion of embryos recovered from superovulated heifers at day 7 after estrus that exhibited normal morphology was reduced by exposure to heat stress for 10 h beginning at the onset of estrus and before insemination at 15-20 h after the beginning of estrus (Putney *et al.*, 1989b). Heat stress can reduce the magnitude of the preovulatory surge



of LH and estradiol-17 $\beta$  (Gwazdauskas *et al.*, 1981; Gilad *et al.*, 1993). There are also direct effects of elevated temperature on nuclear maturation, spindle formation, cortical granule distribution, free radical

formation, mitochondrial function and apoptosis (Payton *et al.*, 2004; Roth and Hansen, 2004, 2005; Ju *et al.*, 2005; Soto and Smith, 2009; Andreu-Vázquez *et al.*, 2010; Nabenishi *et al.*, 2012).

**A**



**B**

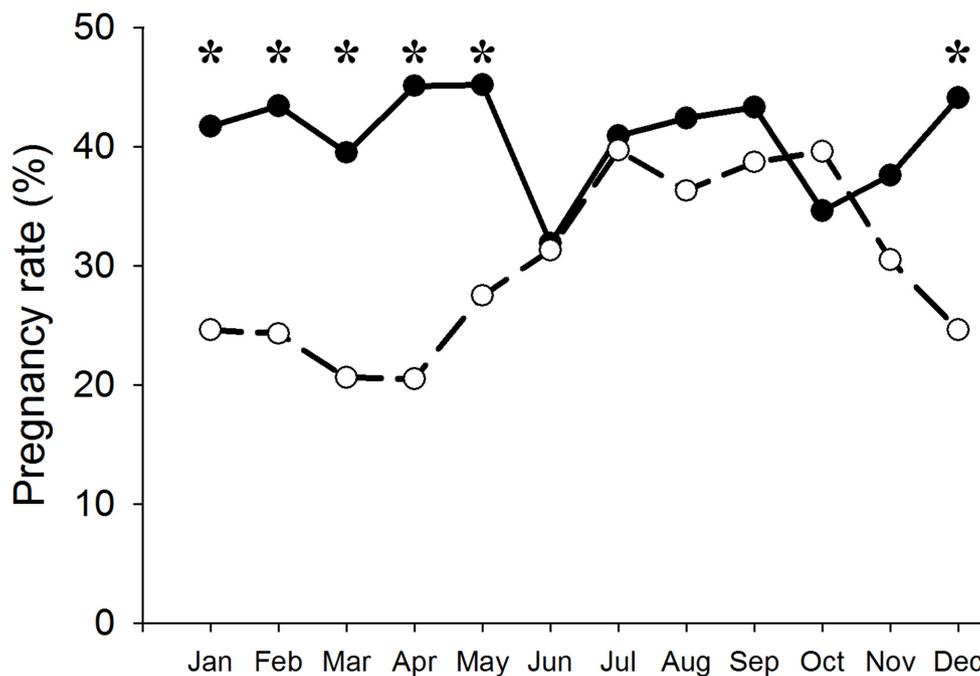


Figure 1. Examples of the effectiveness of embryo transfer for improving fertility in the summer in lactating dairy cows. Data in Panel A are from various experiments in the summer in Florida or Texas. Abbreviations are as follows: AI: artificial insemination; EG, frozen in ethylene glycol; F, fresh; Gly, frozen in glycerol; IVFET, embryo transfer with an in vitro produced embryo; MOET, multiple ovulation embryo transfer; TAI, timed artificial insemination; TET-IVF, timed embryo transfer with an in vitro produced embryo; VIT, vitrified. The numbers in the graph represent the day of gestation at which pregnancy diagnosis was carried out. Note that data from Stewart *et al.* (2011) represent calving rates. Panel B represents data from a commercial dairy in Brazil in which cows were either inseminated (open circles) or received an embryo produced by superovulation (closed circles). Asterisks represent months in which pregnancy rate was different between AI and ET. The data are from Rodrigues *et al.* (2004).



### *Effects of elevated temperature on the embryo*

When first formed, the preimplantation embryo is very susceptible to elevated temperature. Developmental competence of the zygote and two-cell embryo can be compromised by exposure to elevated temperature *in vitro* (Edwards and Hansen, 1997; Sakatani *et al.*, 2004, 2012). Disruption of developmental competence involves reduced protein synthesis, swelling of mitochondria, and cytoskeletal changes characterized by movement of organelles towards the center of the blastomere (Edwards *et al.*, 1997; Rivera *et al.*, 2003). Generation of free radicals occurs in response to culture at elevated temperature, at least in embryos at day 0 and 2 after insemination (Sakatani *et al.*, 2004) and oxidative damage to macromolecules in the embryo could compromise development.

Soon after the two-cell stage, the bovine embryo becomes more resistant to elevated temperature. Development of four to eight-cell embryos can be compromised by heat shock but to a lesser extent than for two-cell embryos (Edwards and Hansen, 1997). By the morula stage of development, exposure of cultured embryos to elevated temperatures has little effect on development (Edwards and Hansen, 1997; Sakatani *et al.*, 2004; Eberhardt *et al.*, 2009; Sakatani *et al.*, 2012). *In vivo*, heat stress reduced blastocyst yield from superovulated cows when occurring at day 1 after estrus but not when occurring at day 3, 5, or 7 after estrus (Ealy *et al.*, 1993).

### *Mechanisms for acquisition of thermotolerance*

Acquisition of thermotolerance by the bovine embryo is coincident with onset of embryonic genome activation, which occurs at the 8-16 cell stage (Memili and First, 2000). However, there are several lines of evidence to indicate that it is not the capacity to synthesis thermoprotective molecules that make the embryo so sensitive to elevated temperature early in development. Perhaps by virtue of inheritance from the oocyte, steady-state amounts of mRNA for *HSPA1A* (heat shock protein 70), *HSP90AA1* (heat shock protein 90) and *SOD1* (superoxide dismutase) are actually higher in the one-cell or two-cell embryo than the morula (Fear and Hansen, 2011; Sakatani *et al.*, 2012; see Fig. 2). New synthesis of heat shock protein 70 in response to elevated temperature can also occur in the two-cell embryo (Edwards *et al.*, 1997; Chandolia *et al.*, 1999).

Recently, it was shown that exposure of morulae to 40°C did not cause a large increase in expression of genes involved in the heat shock protein response. Transcript abundance following heat shock increased for only 4 of 68 genes associated with the heat shock response (Sakatani *et al.*, 2013). Since the main intracellular signal for transcription of heat shock protein genes is denatured protein (Calderwood and Gong, 2012), it is possible that thermotolerant embryos undergo less protein denaturation, and perhaps less cellular

damage in general, than thermosensitive embryos. One reason might be a change in the balance between reactive oxygen species (ROS) generation and antioxidant protection. Culture at elevated temperature increased production of ROS at days 0 and 2 after fertilization but not at days 4 and 6 (Sakatani *et al.*, 2004). The cytoplasmic antioxidant glutathione is at its lowest during the two to eight-cell stages and increases thereafter (Lim *et al.*, 1996).

### *Rationale for ET during heat stress*

Changes in thermosensitivity of the oocyte and embryo to heat shock and what is known about the molecular basis for these changes is illustrated in Fig. 3. As has been outlined in the preceding paragraphs, the oocyte is susceptible early in the process of folliculogenesis and continues to be through the period of oocyte maturation. The embryo also begins its existence in a state that is very susceptible to heat shock. By the 8-16 cell stage, however, the embryo gains resistance to elevated temperature, possibly because of alterations in the balance between ROS production and antioxidant defenses. Given this prolonged period of sensitivity of the oocyte and embryo to heat shock, it is not surprising that cooling cows for short periods of time coincident with ovulation have had only limited impact on fertility (Stott and Wiersma, 1976; Wise *et al.*, 1988b; Ealy *et al.*, 1994). Hormonal treatments have also been unsuccessful at improving fertility during heat stress (see Hansen, 2011 for review). Their ineffectiveness is also probably tied to the broad period of time in which the oocyte and early embryo are susceptible to disruption by heat shock. Hormonal treatments that might mitigate some effects of heat stress cannot reverse others. For example, treatment with GnRH to turnover follicles could conceivably remove a follicle damaged by heat stress but follicles that emerge from the growing pool after GnRH treatment would also have been damaged by heat stress.

Embryos are typically transferred into recipient females when they have reached the morula or blastocyst stages of development, typically at day 7 after ovulation. Embryos used for embryo transfer have escaped harmful consequences of heat stress on the oocyte and embryo, either because they were collected in the cool season, produced *in vitro*, or represent the fraction of oocytes and embryos capable of continued development after heat shock. Moreover, exposure to elevated temperature while developing in the recipient female is unlikely to affect an embryo selected for transfer because the embryo is at a stage of development that is resistant to elevated temperature. The bypassing of effects of heat stress on the oocyte and embryo combined with the placement of a thermoresistant embryo in the uterus means that pregnancy rates with embryo transfer during heat stress can be equivalent to those after artificial insemination during cool weather (Fig. 1B; Rodrigues *et al.*, 2004). Similarly, there was no difference in pregnancy rate in



lactating recipients between summer and winter (Putney *et al.*, 1988b; Drost *et al.*, 1999; Rodrigues *et al.*, 2004; Loureiro *et al.*, 2009).

A word of caution is appropriate. There are two papers with lactating Holsteins that would suggest that, at least under certain circumstances, heat stress can compromise pregnancy establishment in embryo transfer recipients. Working in Brazil with embryos produced by superovulation, Vasconcelos *et al.* (2006) found that rectal temperature at the time of transfer was inversely related to pregnancy rate at days 25 and 46 of gestation, and positively related to pregnancy loss between those

times. Also, Block and Hansen (2007) found seasonal variation in pregnancy rate in Florida using embryos produced *in vitro*. The pregnancy rate at day 45 of gestation was 28% in the cool season and 18% in the warm season. The seasonal effect could be abolished, however, if embryos had been produced in culture in the presence of insulin-like growth factor 1 (IGF-1). In that case, pregnancy rates were 23% in the cool season and 49% in the warm season. Treatment with IGF-1 can make embryos resistant to heat shock (Jousan and Hansen, 2007) and there might be variation between embryos in the degree of thermotolerance at the blastocyst stage.

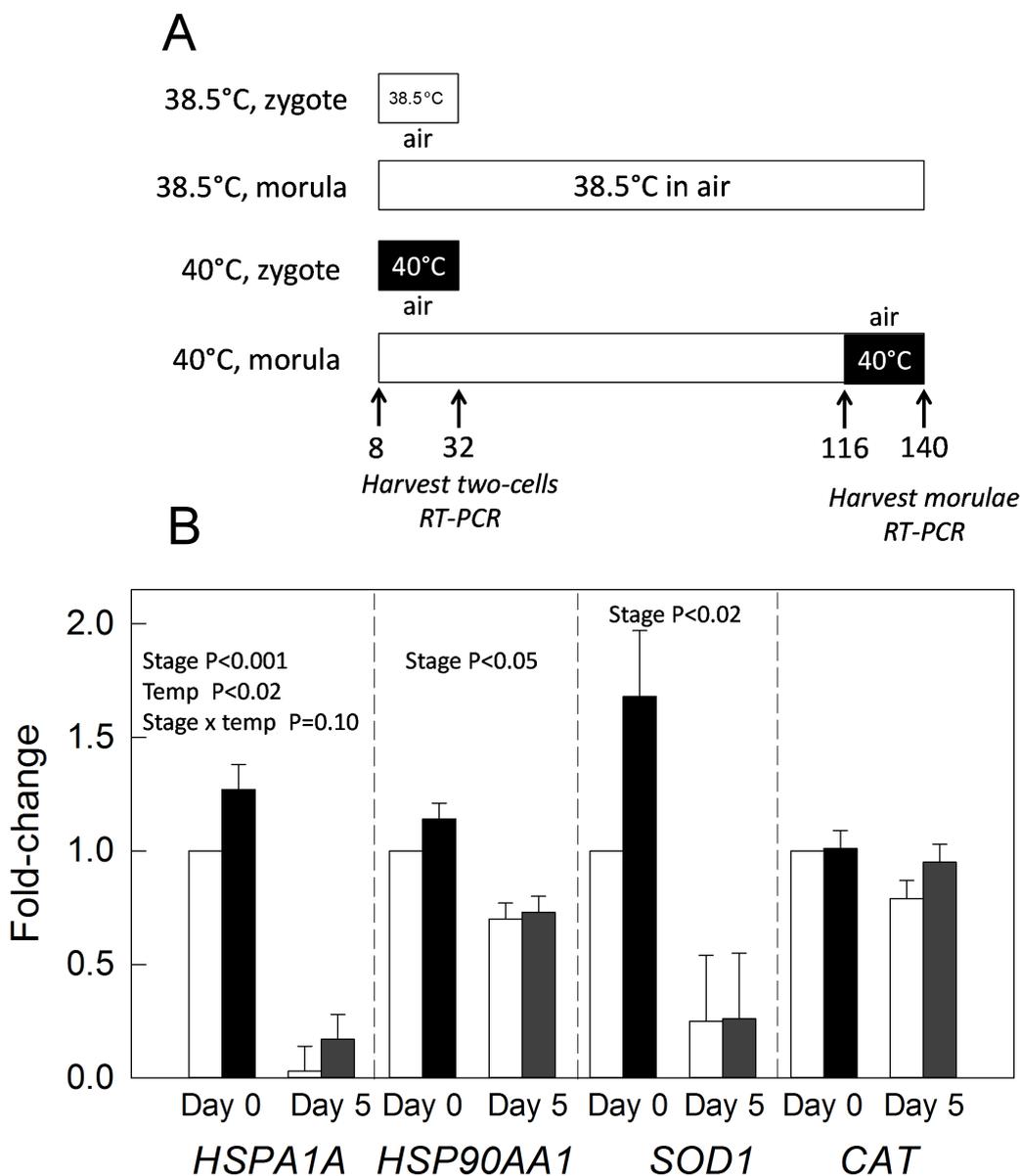


Figure 2. Effects of heat shock on expression of genes involved in cellular thermotolerance in preimplantation bovine embryos. Embryos were collected at the zygote (day 0) or morula stage (day 5) and cultured for 24 h at 38.5°C (open bar) or 40°C (black bar; Panel A). Thus, day 0 embryos had reached the two-cell stage at the time embryos were collected for analysis of RNA. Gene expression is presented in Panel B. The figure is reproduced from *Journal of Dairy Science*; Sakatani *et al.* (2012) with permission.

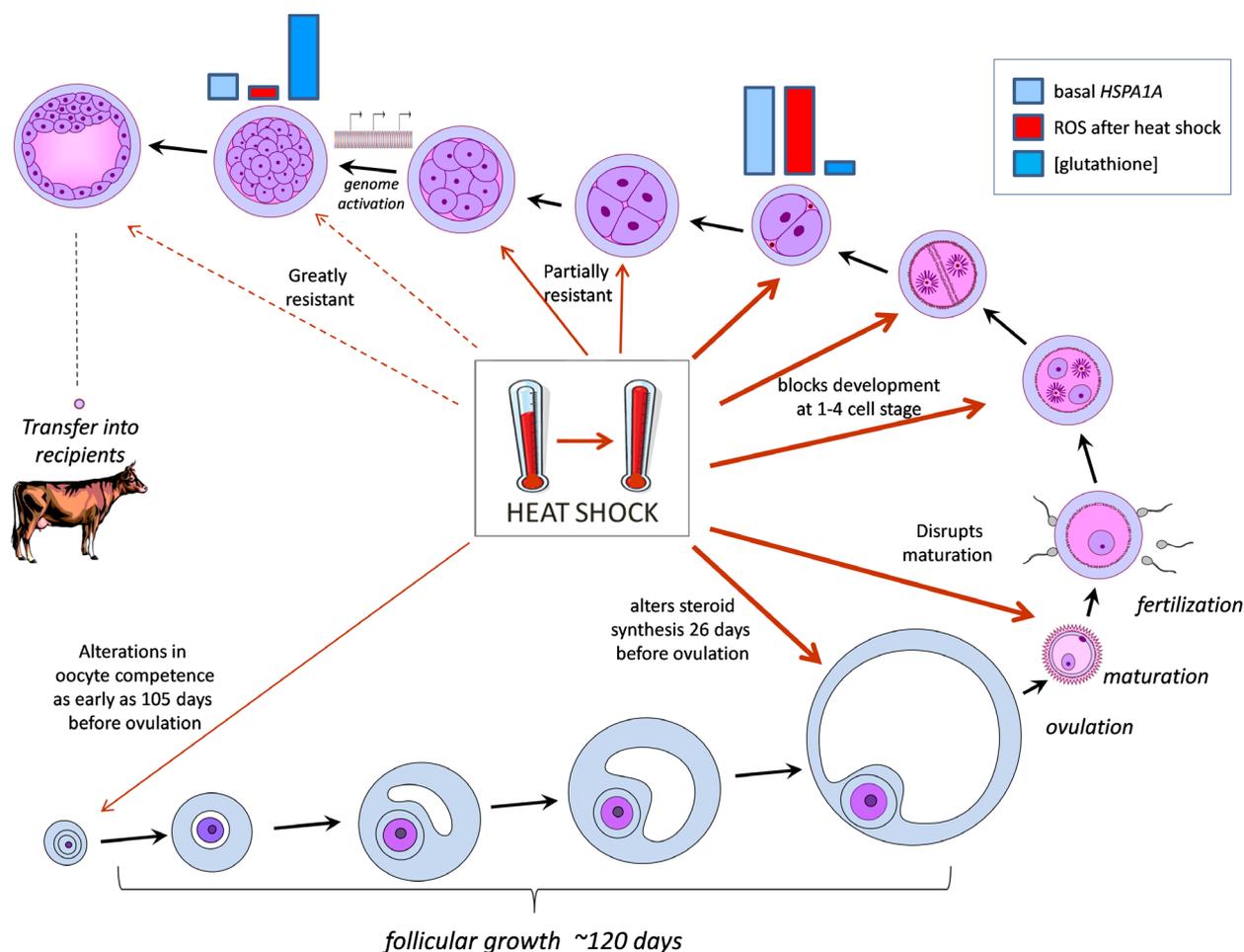


Figure 3. Diagram illustrating the timing of heat shock effects on events leading to blastocyst formation. Heat shock can affect oocyte competence for fertilization and development during follicular development. It is not known how early in the process of folliculogenesis that heat stress is disruptive to oocyte development but results from one paper suggests actions could occur as early as 105 days before ovulation. Oocyte maturation is also compromised by heat shock. The early cleavage-stage embryo remains susceptible to heat shock but thermal tolerance increases by the morula stage. Acquisition of thermotolerance occurs coincident with activation of the embryonic genome. Nonetheless, transcript abundance for some key cytoprotective molecules, including *HSPA1A*, is actually higher at the two-cell stage than at the morula stage. In contrast, the embryo is more susceptible to damage by reactive oxygen species (ROS) early in development. The production of ROS in response to heat shock is greater at the two-cell stage than in the morula. Furthermore, intracellular concentrations of the antioxidant glutathione are low at this stage. Finally, note that by the time an embryo is selected for embryo transfer, typically at the blastocyst stage, it has bypassed damage caused by heat shock during follicular growth or early embryonic development and has also acquired biochemical systems that protect it from elevated temperature. As a result, effects of heat stress in embryo transfer recipients is reduced or absent as compared to cows bred by natural or artificial insemination.

#### Experience with use of antioxidants to protect embryos from heat shock

Exposure of maturing oocytes (Nabenishi *et al.*, 2012) and early cleavage-stage embryos increases production of ROS (Sakatani *et al.*, 2004). This action of heat shock is probably a reflection of the increase in cellular metabolism caused by elevated temperature so that reactions that generate free radicals are increased. *In vivo*, as well, there is one report indicating increased

oxidative stress associated with heat stress in Holstein cows during the transition period (Bernabucci *et al.*, 2002). Results such as these suggest that it might be possible to enhance antioxidant defenses and thereby reduce effects of heat stress on fertility. Unfortunately, most attempts to do so have failed including injections of vitamin E with or without selenium (Ealy *et al.*, 1994; Paula-Lopes *et al.*, 2003a) and injection of  $\beta$ -carotene (Aréchiga *et al.*, 1998b). One reason may be that periodic administration of antioxidants may not be sufficient to



continuously protect the oocyte and embryo from ROS induced by elevated temperature. Fertility was improved by antioxidant supplementation in the one study in which the antioxidant was fed. In particular, Aréchiga *et al.* (1998a) found that feeding cows supplemental  $\beta$ -carotene at a rate of 400 mg/day from about day 15 after calving increased the proportion of cows that were pregnant at 90 days postpartum during the summer but not during the winter. There was no effect of treatment on pregnancy rate at first service so the supplemental  $\beta$ -carotene either improved fertility after first service or estrus detection rate. Recently, Garcia-Ispierto *et al.* (2012) found that administration of melatonin implants beginning at 220 days of gestation to cows during the summer reduced interval to conception in the subsequent postpartum period and decreased the incidence of cows experiencing >3 breedings per conception. Melatonin has antioxidant properties in the follicle (Tamura *et al.*, 2013) and had earlier been found to reduce effects of heat stress in mice (Matsuzuka *et al.*, 2005b).

Free radical chemistry is complex. Some antioxidants function in the water-soluble fraction of the cell and others in the lipid-soluble fraction. Moreover, molecules that act as antioxidants in certain conditions can function as prooxidants in others (Gutteridge and Halliwell, 2010). We know little about the distribution of antioxidants in the reproductive tract and it may be that some are more likely to enter the oocyte or embryo than others. Two antioxidants have been reported to protect embryos from heat shock in culture (anthocyanin; Sakatani *et al.*, 2007), and dithiothreitol (Castro e Paula and Hansen, 2008), whereas vitamin E (Paula-Lopes *et al.*, 2003a), glutathione (Ealy *et al.*, 1995), and glutathione ester (Ealy *et al.*, 1995) were not thermoprotective. There is not yet enough known about the approach to antioxidant supplementation that is the most likely to be effective for improving fertility during heat stress. The recent encouraging results with melatonin (Garcia-Ispierto *et al.*, 2012) deserve further research, including whether fertility-promoting effects are seen in the absence of heat stress.

### Genetic selection for genes that confer cellular thermoprotection

It has long been known that there are genes in cattle that contribute to maintenance of body temperature during heat stress. Thus, certain breeds of beef (Hammond *et al.*, 1996) and dairy cattle (Srikandakumar and Johnson, 2004) are better able to regulate body temperature during heat stress than others. Even in the Holstein, rectal temperature during heat stress is heritable, with estimates of 0.17 (Dikmen *et al.*, 2012). Thus, genetic improvement in resistance to heat stress is possible using genetic selection or crossbreeding.

There are also breed differences in cellular responses to elevated temperature. Nelore, Brahman, and Romosinuano embryos are more resistant to the disruptive effects of elevated temperature on development than Angus or Holstein embryos (Fig. 4). In addition, previous exposure to heat shock tended to reduce ability of Angus blastocysts to establish pregnancy after transfer into recipients whereas there was no effect for Nelore embryos (Silva *et al.*, 2013). Breed differences in thermotolerance have also been observed in endometrium (Malayer and Hansen, 1990) and lymphocytes (Kamwanja *et al.*, 1994; Paula-Lopes *et al.*, 2003b).

In crossbred embryos, it was the breed of oocyte and not breed of spermatozoa that determined whether embryos exhibited increased thermotolerance (Block *et al.*, 2002; Satrapa *et al.*, 2011). For example, embryos produced by fertilization of Brahman oocytes with Angus semen were more resistant to heat shock than embryos produced by fertilization of Holstein oocytes with Angus semen (Block *et al.*, 2002). In the same experiment, there were no differences in thermotolerance of Brahman x Holstein and Angus x Holstein embryos. One explanation for this phenomenon is that transcripts that accumulate in the oocyte are responsible for thermotolerance later in development. Alternatively, the genes conferring cellular thermotolerance are paternally imprinted.

An important unanswered question is whether breed differences exist in resistance of the oocyte or early embryo to heat shock. The silencing of transcription in these cells may not allow for expression of genetic differences in thermotolerance. However, if the transcripts that accumulate in the oocyte are responsible for differences in thermotolerance, genetic differences are likely to occur at early stages of development. Transcript abundance for *HSPA1A* and *HSP90AA1* is higher in two-cell embryos than in morulae (Fear and Hansen, 2011; Sakatani *et al.*, 2012).

Identification of the genes controlling cellular thermotolerance or of genetic markers linked to those genes could lead to selection of cattle possessing embryos with increased resistance to disruption by elevated temperature. To date, only one such genetic marker has been identified. Basiricò *et al.* (2011) studied relationship between two SNPs in the 5' untranslated region of the heat shock protein 70 gene and resistance of peripheral blood mononuclear cells from lactating Holsteins to exposure to 43°C for 1 h *in vitro*. Both SNPs affected viability following heat shock. Moreover, the allele that was associated with increased survival also resulted in increased expression of the HSP70.1 gene. It is worth noting that both of these SNPs were related to calving percentage in seasonal-calving Brahman-influenced cows (Rosenkrans *et al.*, 2010).

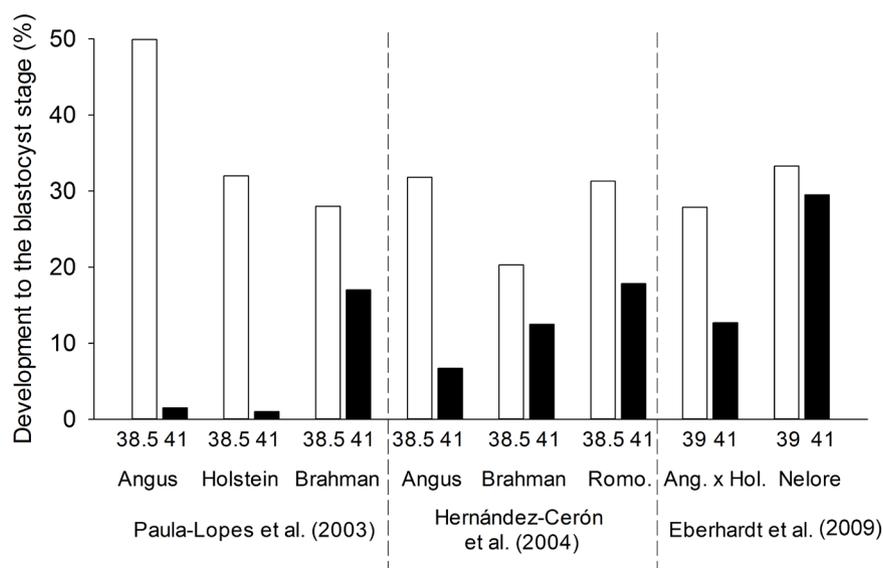


Figure 4. Breed effects on ability of embryos to develop to the blastocyst stage after exposure to heat shock. Embryos were either cultured continuously at either 38.5 or 39°C or were exposed to a heat shock of 41°C on either day 4 (Hernández-Cerón *et al.*, 2004; Eberhardt *et al.*, 2009) or day 5 (Paula-Lopes *et al.*, 2003b) after insemination. The proportion of embryos that became blastocysts was determined. Ang.= Angus, Hol.= Holstein, Romo.= Romosinuano.

### Where do we go from here?

It is crucial that we do go somewhere because all indications are that global climate change will have serious effects on agricultural production in the next 40 years or so (Battisti and Naylor, 2009). With respect to cattle reproduction, it is fortunate that there are prospects for using technology to bypass effects of heat stress on the oocyte and embryo by use of embryo transfer and by increasing resistance of the cow and its embryo to heat shock through nutritional or genetic means. Of these three strategies, embryo transfer is the only one that has been reduced to practice. To become a practical solution for large numbers of farms, additional efforts should be made to reduce the costs of producing a pregnancy by embryo transfer, either by improving efficiency of embryo production or by enhancing the competence of the embryo to develop into a healthy calf. An embryo used for embryo transfer can also be made more valuable by use of sexed semen (Rasmussen *et al.*, 2012), and by genomic testing of the embryo before transfer (Moghaddaszadeh-Ahrabi *et al.*, 2012).

There are encouraging indications that chronic administration of antioxidants such as  $\beta$ -carotene (Aréchiga *et al.*, 1998a) and melatonin (García-Ispierto *et al.*, 2012) can improve fertility. Additional research into development of practical delivery systems is warranted. It may also be fruitful to evaluation fertility-promoting effects of less-well known antioxidants that exist in nature because they may have different properties than the more commonly-studied antioxidants. Two of these, the anthocyanins found in sweet potato (Sakatani *et al.*, 2007) and epigallocatechingallate found in green tea (Roth *et al.*, 2008) have been reported to protect the

embryo (anthocyanins) or oocyte (epigallocatechingallate) from elevated temperature.

Perhaps the most promising way to mitigate the problem of heat stress is to change cattle genetically so that they are better able to regulate body temperature during heat stress and so that oocytes and embryos are better able to cope with elevations in body temperature that do occur during heat stress. The most practical way to do this in the past was by crossbreeding but this practice resulted into the introduction of undesirable genes as well as desirable ones into cattle populations used for production. SNPs for rectal temperature during heat stress have been identified in Holsteins (Dikmen *et al.*, 2013) as well as SNPs for cellular resistance to heat shock (Basiricò *et al.*, 2011). Further advances in livestock genomics, including incorporation of whole genome sequencing into selection schemes (Hayes *et al.*, 2013), should make it easier to identify and select alleles conferring thermotolerance at the whole animal and cellular level. Moreover, it will be possible to use molecular tools like TALENs (Joung and Sander, 2013) to perform genome editing to change the sequence of specific genes and introduce favorable alleles into cattle populations. Some of these alleles could represent new mutations not existing in nature that improve thermotolerance and other traits of importance in a warming world.

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## Equine assisted reproduction and embryo technologies

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### Abstract

Assisted reproductive techniques in the horse have been only recently become available compared to other domestic species, in particular ruminants. The scarce availability of abattoir ovaries and the lack of interest from horse breeders and breed associations, and the anatomical and physiological differences have been the main reasons for this delay. Progressively though, the technology of oocyte maturation *in vitro* has been established especially after the application of ICSI to obtain *in vitro* fertilization. The parallel improvement of oocyte maturation conditions and embryo culture media has increased the rates of embryo development from *in vitro* matured and *in vitro* cultured ICSI embryos from 5-10% in the early studies to up to 26% in the latest under experimental conditions with abattoir derived oocytes. In 2003, the birth of the first cloned foal established the technology of somatic cell nuclear transfer. The largest set of data on non-surgical embryo transfer of *in vitro* produced embryos, from ICSI of *in vitro*-matured Ovum Pick Up (OPU) oocytes, and from somatic cell nuclear transfer, has been obtained in our laboratory. In the clinical context, where OPU and ICSI are applied for the treatment of female and or male infertility, the yield of embryos has been lower compared to experimental conditions. In conclusion, the basic procedures have been established for the use of assisted reproduction and somatic cell nuclear transfer to a degree suitable for clinical applications and the results have been replicated in several laboratories around the world.

**Keywords:** cryopreservation, embryo culture, ICSI, oocyte maturation, sexing, somatic cell nuclear transfer.

### Introduction

Besides classical reproduction techniques such as artificial insemination and embryo transfer (Squires *et al.*, 2003), advanced reproductive biotechnologies like oocyte recovery and maturation, oocyte transfer, *in vitro* fertilization, embryo culture, embryo manipulation and nuclear transfer were first established in ruminants and served as a translational model both for the human and the horse.

The flow of knowledge from the animal field that has advanced human assisted reproduction in the

past is now reverted and some information from the human field is benefiting the animals and in particular the horse with intracytoplasmic sperm injection (ICSI), pre-implantation genetic diagnosis, vitrification of oocytes and embryos, etc. As a matter of fact the scope for application of ART in the horse is very much similar to the human: to reproduce individuals by overcoming physiological, pathological or technical barriers to reproduction.

The progress in advanced assisted reproduction in the horse has been continuous although at an irregular pace compared with other domestic species and major developments occurred only in the last 12 years. In this paper we will review the recent progress in assisted reproduction in the horse with the main emphasis on the procedures of oocyte recovery and maturation, ICSI, *in vitro* embryo production, pre-implantation genetic diagnosis, cloning, and cryopreservation.

### Oocyte recovery

#### *Ovum Pick Up of matured oocytes*

The use of assisted reproduction in the horse is limited to animals with fertility problems, both from female and male origin or those engaged in sporting activities to produce in most cases a limited number of embryos and offspring. As a consequence, a number of techniques, including laparotomy, colpotomy or blind aspiration through the paralumbar fossa, have been attempted to recover oocytes from live donors without compromising their reproductive and sporting abilities. Oocytes can be collected from the preovulatory follicle 24 h after administration of hCG to a follicle that has reached at least 35 mm and the donor showing signs of uterine edema. Only mild superovulatory treatment has benefited oocyte recovery (Altermatt *et al.*, 2009). Ovulation normally takes place 36-40 h after hCG. Therefore oocytes collected at 24 h or later have resumed meiosis, have an expanded cumulus that facilitate recovery but require 16 h of additional culture prior to transfer. Oocytes are more frequently recovered by ultrasound guided transvaginal follicular aspiration using a double lumen 12G needle (Carnevale *et al.*, 2005). After recovery from the donor mare the oocytes complete their maturation *in vitro* and then can be surgically transferred to inseminated recipients

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(Carnevale, 2004) whose preovulatory oocyte has been aspirated or can be subjected to ICSI for being transferred surgically to the oviduct of a synchronized recipient. In a clinical setting the success rate of establishing a pregnancy with oviduct transfer is in the range of 40% (Carnevale, 2004).

#### *Ovum Pick Up of immature oocytes*

Oocytes from antral follicles can be recovered as it is done in cattle (Galli *et al.*, 2001), defined as ultrasound guided transvaginal oocyte recovery or Ovum Pick Up (OPU). Similarly to its use in other species, this technology, developed in human medicine, started in the early nineties and has been subsequently refined (McKinnon *et al.*, 1998; Cochran *et al.*, 2000). This technology has been proven safe and repeatable in cattle and has the advantage of not requiring any hormonal stimulation of the donor. This aspect is of considerable importance in the horse because of the limitations of superovulation but also because it is difficult to handle large ovaries afterward for oocyte recovery. Oocyte recovery from immature follicles in the horse requires vigorous flushing and scraping of the follicle. There are several data reported in the literature for the recovery of immature oocyte recovery (Bogh *et al.*, 2003; Vanderwall *et al.*, 2006) but very limited data to assess of the developmental potential of such oocytes for producing horse embryos on a routine basis.

In our laboratory we have carried out a large study on the developmental capacity of immature oocytes recovered by OPU from both experimental and commercial donors within a commercial OPU and we currently offer this service to horse breeders (Colleoni *et al.*, 2007). The data reported in Table 1 refer to repeated OPU collections from donors of various breeds within a clinical OPU programme. Donors are monitored by ultrasound to select the best time for oocyte collection when there are several antral follicles and preferably the absence of a dominant follicle. This situation is particularly favorable during the transition season (no dominant follicle with many medium antral follicles). We do not collect normally oocytes from mares that have less than 5-7 follicles and we wait for

more follicles to grow. The interval between collections is 2-4 weeks depending on the mares. Mares can be subjected to repeated collections without any side effect if the procedures are performed correctly (Mari *et al.*, 2005). We sedate the donor with detomidine and give epidural anesthesia to avoid the contraction of the rectum during ovary handling and catheterize the bladder. At the end of the procedure the donor is treated with antibiotic for 3 days. Other programs have reported fixed biweekly schedule (Jacobson *et al.*, 2010).

Table 1. Average embryo production from an OPU session at Avantea in a clinical setting. Donors are monitored by ultrasound scanning to select the appropriate moment to avoid the presence of pre-ovulatory follicles in favor of medium sized follicles (10-20 mm). Matured oocytes are subjected to ICSI and cultured for 7-8 days to the blastocyst stage when embryos are cryopreserved.

	Number	Rate (%)
Follicles	16.1	--
Oocytes	10.8	67.3
Matured oocytes	6.9	63.7
Cleaved oocytes	3.7	54.3
Blastocyst	0.5	7.4

Data from approximately 700 OPU sessions.

#### *Oocyte collection from ovaries collected at the abattoir or after euthanasia*

The collection of oocytes requires the dissection of the connective tissue enclosing the ovary, the incision of visible follicles and scraping of the inside follicle wall with a curette and extensive flushing to detach the cumulus-oocyte complexes (COCs). In the horse, two populations of COCs can be identified and oocytes with expanded cumulus mature normally and have normal developmental competence contrary to what is seen in other species. Table 2 presents the data from a large study conducted in our laboratory that gives an exact measure of the efficiency of oocyte collection from abattoir ovaries and the expected maturation rate from compact and expanded COCs.

Table 2. Maturation competence of horse oocytes derived from expanded or compact COCs.

No. ovaries	No. follicles (per ovary)	No. COCs expanded (per ovary)	No. COCs compact (per ovary)	No. COCs matured after IVM		No. COCs degen. after IVM	
				Expanded (%)	Compact (%)	Expanded (%)	Compact (%)
603	3204	590	1672	354	855	177	558
	5.3	1	2.8	(60.0%) <sup>a</sup>	(51.1%) <sup>b</sup>	(30.0%)	(33.4%)

Chi square test: values with different letters differ ( $P < 0.05$ ). Galli *et al.* (2007).



### Oocyte maturation in vitro and conventional *in vitro* fertilization

The first successful report of *in vitro* maturation of horse oocytes was that of Fulka and Okolski (Fulka and Okolski, 1981). The first embryo production from *in vitro* matured horse oocytes was reported in 1989 (Zhang *et al.*, 1989) when oocytes collected at the abattoir were matured *in vitro* and, after transfer to the oviducts of inseminated mares and recovery by uterine flushing 7 days later, had developed to the blastocyst stage. Oocytes are matured *in vitro* for 24-28 h at 38.5°C in 5% CO<sub>2</sub>. In our culture conditions, as shown in Table 2, the maturation rate ranges from 51.1 to 60% for compact and expanded COCs respectively. This difference is statistically significant and in agreement with other studies (Hinrichs and Williams, 1997; Hinrichs and Schmidt, 2000; Hinrichs *et al.*, 2005) in which oocytes with expanded cumulus were found more capable to complete maturation than were oocytes with compact cumuli. It is interesting to note that in Table 2 the distribution of the degenerated oocytes is equal between the compact and expanded COCs groups. This is somehow surprising because, in other species, the presence of a compact and healthy cumulus cell layer is considered an indicator for good oocyte morphology. Since no lysis is observed with oocytes collected by OPU from live donors (Fig. 1a and b), this finding implies that the post mortem modifications occurring in the large equine ovaries are responsible for the degeneration of abattoir oocytes. Indeed, when

equine oocytes were placed into maturation immediately after slaughter, the rate of maturation was higher and the rate of degeneration lower than those for oocytes recovered after transport of ovaries to the laboratory (Hinrichs *et al.*, 2005).

Interestingly, only two foals were reported as born from IVF and both were derived from *in vivo* matured oocytes collected by OPU from gonadotropin stimulated donors (Palmer *et al.*, 1991; Bezar, 1992). There is no published report of equine pregnancies derived from both *in vitro* maturation and conventional IVF. Failure of IVF remains a mystery but is probably related to inefficient sperm capacitation (Alm *et al.*, 2001), changes in the zona pellucida (Dell'Aquila *et al.*, 1999; Hinrichs *et al.*, 2002) or to incomplete *in vitro* maturation (Li *et al.*, 2001).

To date, a variety of oocyte maturation conditions has been evaluated using different maturation media comprising TCM199 (Willis *et al.*, 1991; Dell'Aquila *et al.*, 1997; Hinrichs and Schmidt, 2000; Galli *et al.*, 2002b; Lagutina *et al.*, 2005), B2 (Willis *et al.*, 1991) or DMEM/F12 (Galli *et al.*, 2007; Table 3), supplemented with different concentration of serum, hormones or follicular fluid. Recently a paper was published (McPartlin *et al.*, 2009) reporting high *in vitro* fertilization rates utilizing procaine for sperm capacitation, however there was no evidence of sperm penetration and the results were based only on the segmentation of the oocytes that could well be fragmentation or parthenogenetic activation. No embryo development was reported and the findings not replicated in other laboratories.

Table 3. Effect of maturation media on maturation, cleavage, and development rate of ICSI embryos.

Maturation medium	No. oocytes	No. degenerated	No. met. II (%)	No. injected	No. cleaved (% of injected)	No. blastocysts (% of injected)
TCM 199	434	105	205 (47.2%) <sup>a</sup>	191	111 (58.1%) <sup>a</sup>	23 (12.0%) <sup>a</sup>
DMEM/F12	338	71	159 (45.6%) <sup>a</sup>	140	108 (77.1%) <sup>b</sup>	37 (26.4%) <sup>b</sup>

replicates = 6

Chi Square test. Numbers within columns with different letters differ ( $P < 0.05$ ). Galli *et al.* (2007).

### Intracytoplasmic sperm injection (ICSI) and embryo culture

The clinical use of ICSI was developed several years ago to overcome male infertility in human assisted reproduction (Palermo *et al.*, 1992). The technique works so efficiently that it is now the preferred way of *in vitro* fertilization in humans even if it is suggested that sperm selection does not occur in a physiological way and it might carry abnormalities to the offspring. However when data are adjusted for age, twin pregnancies, infertility and other patient related factors ICSI offspring is not different from normal offspring (Palermo *et al.*, 2008, 2012). The application of ICSI to

the horse (Fig. 1c), on the other hand, has overcome inefficient conventional IVF, irrespectively of the fertility of the semen used, resulting in the first pregnancy derived from an *in vitro* matured oocyte (Squires *et al.*, 1996), which was carried successfully to term. This success was followed by a period of variable results until the development of ICSI using the piezo drill, which removed most of the inconsistency of the technique due to a heterogeneous and thick zona pellucida difficult to penetrate with conventional ICSI pipettes, especially in *in vitro* matured oocytes with minimal damage to the oocytes. Moreover the advantage of ICSI is the ability to widen the choice of the stallions to be used, including those with poor sperm motility and reproductive performance *in*



*vivo*. In an original study, Lazzari *et al.* (2002a) compared the developmental capacity of *in vitro* matured oocytes fertilized by ICSI with frozen-thawed stallion semen of different motility and/or different fertility in the field. There was no difference in either cleavage or advanced embryo development rate among oocytes injected with sperm from stallions of good, poor and no fertility in the field, as long as a motile sperm was selected for ICSI (Table 4). These results indicate

that ICSI can allow the use of semen with poor motility or no *in vivo* fertility, provided that a motile sperm cell is selected for injection. In another study (Choi *et al.*, 2006), oocytes injected with immotile sperm isolated from semen subjected to two freeze-thaw cycles were capable of blastocyst development. In a further study the same authors (Choi *et al.*, 2011a) have even used lyophilized sperm and activation with sperm extract to generate offspring.

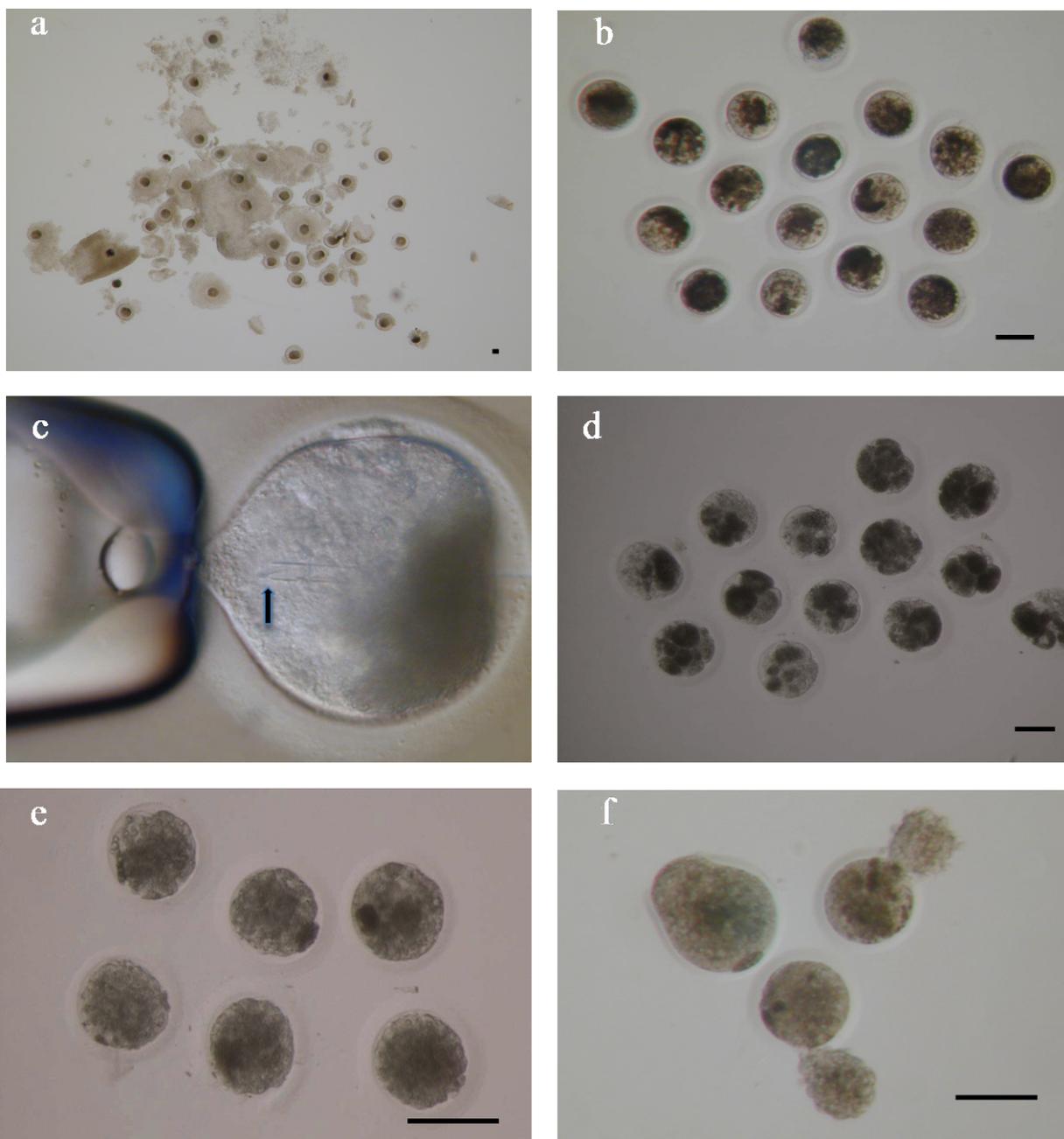


Figure 1. a) Oocytes collected from one OPU session: most of the oocytes display a compact cumulus. b) Denuded oocytes after *in vitro* maturation and selected for the presence of the first polar body ready for ICSI. c) Oocyte being injected with a spermatozoa indicated by the arrow. d) Cleaved embryos 48 h after ICSI. e) Embryos selected for freezing and or transfer on day 7 of development. f) When embryos are cultured *in vitro* for long time (up to day 10+) they begin the extrusion of cells from the hole made with the ICSI pipette.



Table 4. Cleavage and development of horse embryos following ICSI with semen of different motility and fertility post-thawing.

Group of stallions	No. met. II injected	No. Cleaved	Cleavage %	No. comp. morulae and blastocysts	% comp. morulae and blastocysts/injected
A	117	88	75.2 <sup>a</sup>	42	35.9 <sup>a</sup>
B	78	49	62.8 <sup>a</sup>	21	26.9 <sup>a</sup>
C	91	72	79.1 <sup>a</sup>	33	36.3 <sup>a</sup>
D	46	4	8.7 <sup>b</sup>	0	0.0 <sup>b</sup>

Chi square test. Numbers within columns with different letters differ ( $P < 0.05$ ). Lazzari *et al.* (2002).

The progress of *in vitro* maturation and ICSI technology has increased efforts to design suitable culture systems for early cleavage stage embryos (Fig 1d). Many different culture conditions have been reported for preimplantation development of ICSI fertilized horse oocytes, including defined media such as G1.2 (Choi *et al.*, 2002), DMEM-F12 and CZB (Choi *et al.*, 2004) and modified SOF (Galli *et al.*, 2002b). In most of these systems, however, the blastocyst rates remained low, ranging from 4 to 16%. In contrast, the culture of presumptive zygotes following ICSI, *in vivo* either in the mare oviduct or in the surrogate sheep oviduct, allowed much higher development (Galli and Lazzari, 2001; Table 5).

A comparison between the published reports on *in vivo* culture of ICSI early cleavage stage embryos in the oviducts of mares (Choi *et al.*, 2004) or temporary

recipient sheep (Galli *et al.*, 2002a; Lazzari *et al.*, 2002a) and *in vitro* culture in various culture media (see above) clearly demonstrated that the *in vivo* environment supports higher blastocyst development, being approximately 36% of injected oocytes in both the mare oviduct and sheep oviduct. Recently, an *in vitro* culture system has been developed using DMEM/F-12 medium under a mixed gas atmosphere that provides blastocyst development rates similar to those seen *in vivo* (27-38%) (Alm *et al.*, 2008; Jacobson *et al.*, 2010) (Fig 1f). However when cell number counts were compared (Tremoleda *et al.*, 2003) among *in vivo* produced embryos and those produced by *in vitro* culture in a modified SOF medium, both on day 7 of development, *in vitro* produced embryos had significantly lower cell numbers, resembling a day 5 *in vivo* embryo rather than a day 7.

Table 5. Oocyte recovery rate by OPU and effect of *in vitro* or *in vivo* sheep oviduct culture on embryo development.

	No. OPUs	No. oocytes (no. per OPU)	No. met. II (% of oocytes) (no. per OPU)	No. cleaved (% of injected) (no. per OPU)	No. comp. morulae/blastocysts (% of injected) (no. per OPU)
Sheep oviduct culture	20	60 (3.0)	46 (76.7%) (2.3)	41 (89.1%) <sup>a</sup> (2.1)	23 (50.0%) <sup>a</sup> (1.2)
<i>In vitro</i> culture	12	46 (4.1)	36 (73.5%) (3.0)	25 (69.4%) <sup>a</sup> (2.1)	5 (13.9%) <sup>b</sup> (0.4)

Student T test. Numbers within columns with different letters are significantly different ( $P < 0.05$ ). Galli and Lazzari (2001).

### Somatic cell nuclear transfer

The first equine clones, three mules and one horse, were reported in 2003 both from fetal cell (Woods *et al.*, 2003) and from adult cells (Galli *et al.*, 2003). The first horse, a filly, was obtained by nuclear transfer of adult somatic cells, using *in vitro* matured oocytes as recipients of the donor nuclei and *in vitro* culture to the blastocyst stage before non surgical transfer to recipient mares. In that study, the foal was born to the same mare that donated the cells used in the cloning procedure, representing an exclusive example of autologous pregnancy successfully gone to term. Other cloned foals were born (Lagutina *et al.*, 2005; Hinrichs *et al.*, 2006)

by SCNT, demonstrating the reproducibility of this technology. Additionally, two cloned foals were born in 2005 in another laboratory confirming that the cloning technology is now established in horses (Hinrichs *et al.*, 2006) and that it can be done by using *in vitro* matured oocytes. More foals have been produced (Hinrichs *et al.*, 2007) in the same laboratory and in another laboratory (Gambini *et al.*, 2012). Other activities are undergoing in commercial laboratories but there is no published information about their activities and results. Development rate of SCNT embryos is greatly influenced by the cell line (Lagutina *et al.*, 2005) which is well known in other species and varies from 0 to 17%. Oocyte maturation *in vitro* is the only sustainable



source of oocytes for cloning and their quality is as critical as it is for ICSI. We have also tested the effect of changing maturation medium from TCM199 to DMEM-F12 on nuclear transfer (NT) embryo development (Table 6) and we have confirmed the positive results obtained with ICSI embryos. Enucleation of the oocytes and nuclear transfer can be obtained by the zona-free method followed by the electrical fusion of the somatic cells and the oocyte (Lagutina *et al.*, 2007) or by using the piezo-electric manipulator that is used both for enucleation and for the injection of the somatic cell after breaking its cell membrane (Hinrichs *et al.*, 2006). Activation of the reconstructed embryos is performed in our laboratory by using a combination of the two most common chemicals used in other species for induction of parthenogenetic

development: 6-dimethylaminopurine (6-DMAP) and cycloheximide (Lazzari *et al.*, 2002b). Other workers (Hinrichs *et al.*, 2006) have used a combination of injection of sperm extract and culture in 6-DMAP to produce embryos resulting in successful foaling of cloned offspring. Although the development to term of cloned pregnancies is low as in other species, most of the pregnancy losses occur early in gestation (before day 50) thus creating less problems with the recipient management and the numbers of recipients required that can eventually be re-used. Moreover the foaling and foal survival are normal and we do not see the problems reported with cattle, for example of hydrops, placenta hyperplasia, and large offspring syndrome. Most of the foals are normal or require minor assistance at birth (Johnson *et al.*, 2010).

Table 6. Effect of maturation media on maturation, cleavage, and NT embryo development.

Maturation medium	No. oocytes	No. lysated	No. met. II (%)	No. NT embryos	No. cleaved (% of NT)	No. blastocysts (% of NT)
TCM 199	164	35	64 (39.0%) <sup>a</sup>	41	38 (92.7%) <sup>a</sup>	4 (9.8%) <sup>a</sup>
DMEM/F12	166	35	67 (40.4%) <sup>a</sup>	47	46 (97.9%) <sup>a</sup>	13 (27.7%) <sup>b</sup>

Chi Square test. Numbers within columns with different letters differ ( $P < 0.05$ ). Galli *et al.* (2007).

### Semen and embryo sexing and pre-implantation diagnosis

Sexing of semen in the horse has been done using flow cytometric cell sorting based on DNA quantitative differences between X and Y bearing sperm (Garner, 2006). The number of sperm required for a standard dose of artificial insemination cannot be obtained with the current technology, therefore, sexed semen can only be used for a low dose insemination with a fraction of the sperm that are normally used for artificial insemination (Lindsey *et al.*, 2002a, b). Results are much lower when using frozen sex sorted sperm (Clulow *et al.*, 2008). The low fertility, associated with loss of pregnancies (Gibb *et al.*, 2012), does not make it commercially viable for the industry also when used for embryo transfer programs. The natural way to use low number of sperm with poor viability would be ICSI. We have used sex sorted frozen thawed semen with ICSI (Colleoni *et al.*, 2009) and obtained very low cleavage but normal development (overall 10% of the efficiency obtained with the non sexed control semen), pregnancies and live foals. In another report no success was reported using sexed refrigerated semen for ICSI (Samper *et al.*, 2012). An alternative approach is the use of embryo biopsy and PCR for sex determination. Making the biopsy is complicated in the horse by the presence of the capsule. The biopsy is taken by aspiration with a micropipette driven by piezo electric

micromanipulator without compromising the viability (Choi *et al.*, 2010). After recovery of the biopsy composed by few cells, it is subjected to WGA (whole genome amplification). This procedure generates sufficient DNA not only to perform sex determination (Peippo *et al.*, 1995; Bannasch *et al.*, 2007) but also for pre-implantation genetic diagnosis (Hinrichs and Choi, 2012).

### Embryo cryopreservation

To date most of the equine embryos are transferred fresh or after cooling at 4°C for up to 24 h and very few are cryopreserved. The main obstacle to this development has been the poor success with embryos larger than 300 µm that are normally recovered at flushing. Collecting embryos earlier so that they are smaller than 300 µm significantly reduces the recovery rate. Smaller embryos stand very well classic slow freezing procedures with 10% glycerol but a freezing apparatus is required (Stout, 2012). Vitrification can also be used for cryopreservation; again smaller embryos survive better than larger ones (Eldridge-Panuska *et al.*, 2005), a freezing machine is not required, and kits for vitrification are available on the market. In an attempt to improve the survival of large embryos (Choi *et al.*, 2011b) it was noticed that collapsing the blastocoele cavity, as it happens when taking a biopsy for sexing, improved survival, this is



probably due to the reduction of the large amount of fluid contained in the cavity that cannot be permeated by the cryoprotectant in normal conditions. In this respect *in vitro* produced embryos through ICSI can be selected at the appropriate stage of development, before they get too large, for slow freezing. Pregnancy rate have been in the range of 50 to 60%, making *in vitro* embryo production even more attractive for this advantage (Colleoni *et al.*, 2007).

### Embryo transfer, pregnancies, and offspring

The true measure of oocyte developmental competence is the ability to generate viable offspring following the transfer, even though a percentage of failures can also be attributed to the recipient itself. Therefore, one of our research priorities, given also its practical application, was to show that the embryos produced with the techniques described above had normal developmental competence and therefore validated the steps involved including *in vitro* maturation, ICSI, embryo culture, and conventional cryopreservation in 10% glycerol. After 10 years of work in clinical practice using OPU and ICSI we can say that *in vitro* produced embryos have high survival rate after transfer with high pregnancy rates even after freezing and thawing. Pregnancy rates vary from 40 to 60% with a foaling rate between 35 to 50%. The transfer of *in vitro* produced embryos is done in recipients 5 days after ovulation since they are at the early blastocyst stage at day 7 of *in vitro* culture and this chronologically asynchronous transfer gives time to the embryo to “catch up” the delay that is present in development after *in vitro* culture. Pregnancy losses and perinatal mortality in our experience has been comparable to that obtained after artificial insemination. We never observed the phenotype of the LOS (large offspring syndrome) occasionally reported for ruminants.

### Conclusions

Oocyte developmental competence is clearly the key requirement for all the technologies that have been described and the main bottleneck. Oocyte transfer, briefly described here, relies on *in vivo* matured oocytes and provides very high pregnancy rates but in small numbers. In addition, the animal welfare and economic issues involved, have given a strong thrust to the development of *in vitro* procedures for oocyte maturation. ICSI has allowed testing of the relationship between *in vivo* and *in vitro* fertility of stallions, demonstrating that even semen with poor fertility in the field can be successfully used for embryo production *in vitro* (Lazzari *et al.*, 2002a). However, sperm too play a significant role in determining embryo development and there are also in this case, as for the oocytes, extreme cases of infertility. Blastocyst production rate is influenced not only by culture environment, *in vivo*

versus *in vitro*, but also by oocyte maturation conditions. The change of maturation medium from conventional TCM199 to DMEM-F12 has a pronounced effect on blastocyst rate in our laboratory, suggesting that probably there is still room for improvement in *in vitro* maturation conditions of equine oocytes. The culture environment, *in vivo* and *in vitro*, has been extensively tested and although the *in vivo* methods, either the mare oviduct or the surrogate sheep oviduct, have proven superior, the *in vitro* culture systems have improved considerably allowing high rates of blastocyst development. A common feature of all the *in vitro* culture systems used is that the embryos tend to be retarded with fewer cell numbers than expected. This finding has suggested that we should allow some time for the day 7-8 IVP embryos to catch up with normal development by transferring them into recipient mares 5 days after ovulation instead of 7 days. Pregnancy rates, following non-surgical transfer of ICSI embryos, have been acceptable for clinical use even after cryopreservation. Similarly, cloned embryos produced from *in vitro* matured oocytes are able to establish pregnancies after *in vitro* culture to the blastocyst stage followed by non-surgical transfer. All this work opens the way to the successful clinical application of assisted reproduction technologies in the horse. However, until the industry takes a more open attitude towards these technologies and allows registration of the animals obtained even by the more controversial techniques such as cloning (Church, 2006), many of these technologies will remain at the experimental stage.

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## Evaluation and classification of bovine embryos

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### Abstract

Bovine Embryo transfer has been used widely to reproduce the most valuable females in the herd, with about 750,000 embryos produced annually from superovulated donors and more than 450,000 embryos produced using in vitro techniques. Furthermore, embryos are the safest and most cost effective alternatives to move genetics internationally because of their low risk of transmitting diseases. One of most important factors associated with the success and widespread application of this technology is evaluation of the embryos before freezing and/or transfer to a recipient. Embryos are usually classified based on a number code system for their stage of development (1 to 9) and for their quality (1 to 4). The basic principles of embryo evaluation are briefly described.

**Keywords:** classification, embryo, freezable, IETS, transferable.

### Introduction

Although the first embryo transfers were performed in rabbits by Walter Heape in 1890, the commercial bovine embryo transfer industry evolved during the early 1970's, with the introduction of continental breeds of cattle into North America (Betteridge, 2003). Since that time, the use of embryo transfer technology in cattle breeding has continued to increase. Nowadays, more than 750,000 embryos are produced annually from superovulated donors and more than 450,000 embryos are produced using in vitro techniques (Stroud, 2012). Embryos are moving around the world because embryos have a relatively low risk of transmitting diseases. In order to do that, embryo transfer teams must adhere to the recommendations of the Manual of the International Embryo Transfer Society "*A procedural guide and general information for the use of embryo transfer technology emphasizing sanitary procedures*" (Stringfellow and Givens, 2010) which has become the reference source for sanitary procedures used in embryo export protocols. Furthermore, one of most important factors associated with the success and widespread application of this technology is the

evaluation of the embryos before freezing and/or transfer to a recipient. In this manuscript the basic principles of embryo evaluation will be briefly described.

### Embryo evaluation

Evaluation of bovine embryos is normally done with a stereomicroscope at 50 to 100X magnification, with the embryo in a small holding dish. It is also necessary to "roll" the embryo on the bottom of the dish so as to view the embryo and zona pellucida from different perspectives. The overall diameter of the bovine embryo is 150 to 190 µm, including a zona pellucida thickness of 12 to 15 µm. The overall diameter of the embryo remains virtually unchanged from the one-cell stage until blastocyst stage. The best predictor of an embryo's viability is its stage of development relative to what it should be on a given day after ovulation. An ideal embryo is compact and spherical. The blastomeres should be of similar size with even color and texture. The cytoplasm should not be granular or vesiculated. The perivitelline space should be clear and contain no cellular debris. The zona pellucida should be uniform; neither cracked nor collapsed and contains no debris on its surface.

It is important to be able to recognize the various stages of development and to compare them with the developmental stage that the embryo should be for the day of the estrous cycle that donors are collected (i.e. usually day 7 after standing estrus). The decision as to whether an embryo is worthy of transfer or freezing and whether the embryo is eligible for export will rely on the expertise and experience of the person that evaluates the embryos. Standardized coding systems for use in describing the stage of development and quality of the embryo are described in Chapter 9 and illustrated in Appendix D of the IETS Manual. The code for stage of development is numeric, ranging from "1", an unfertilized oocyte or a 1-cell embryo to "9", expanding hatched blastocyst. Normally, embryos are collected 7 days after estrus for cryopreservation or transfer and the IETS Manual's standards for the stages likely to be encountered at that time are described below.

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### Stages

- Morula (Stage code 3): A mass of at least 16 cells. Individual blastomeres are difficult to discern from one another. The cellular mass of the embryo occupies most of the perivitelline space.
- Compact morula (Stage code 4): Individual blastomeres have coalesced, forming a compact mass. The embryo mass occupies 60 to 70 % of the perivitelline space.
- Early blastocyst (Stage code 5): An embryo that has formed a fluid-filled cavity or blastocele and gives a general appearance of a signet ring. The embryo occupies 70 to 80% of the perivitelline space. Early in this stage the embryo may appear of questionable quality because it is difficult to differentiate inner cell mass from trophoblast cells at this time.
- Blastocyst (Stage code 6): Pronounced differentiation of the outer trophoblast layer and of the darker, more compact inner cell mass is evident. The blastocele is highly prominent, with the embryo occupying most of the perivitelline space. Visual differentiation between the trophoblast and the inner cell mass is possible at this stage of development.
- Expanded blastocyst (Stage Code 7): The overall diameter of the embryo dramatically increases, with a concurrent thinning of the zona pellucida to approximately one-third of its original thickness.
- Hatched blastocyst (Stage code 8): Embryos recovered at this developmental stage can be undergoing the process of hatching or may have completely shed the zona pellucida. Hatched blastocysts may be spherical with a well defined blastocele or may be collapsed. Identification of hatched blastocysts can be difficult unless they re-expand when the signet ring appearance is again obvious.

### Quality

The codes for embryo quality is also numerical and are based on morphological integrity of embryos. The codes for embryo quality range from “1” to “4” as follows:

- Code 1: Excellent or Good. The embryos have a symmetrical and spherical mass with individual blastomeres that are uniform in size, color, and density. This embryo is consistent with its expected stage of development. Irregularities should be relatively minor, and at least 85% of the cellular material should be an intact, viable embryonic mass. This judgment should be based on the percentage of embryonic cells represented by the extruded material in the perivitelline space. The zona pellucida should be smooth and have no concave or flat surfaces that might cause the

embryo to adhere to a petri dish or a straw. Code 1 embryos survive well to the freezing/thawing procedure and some practitioners call them “Freezable embryos”. Grade 1 embryos are also those recommended for international trade.

- Code 2: Fair. These embryos have moderate irregularities in the overall shape of the embryonic mass or in size, color, and density of individual cells. At least 50% of the embryonic mass should be intact. Survival of these embryos to the freezing/thawing procedure is lower than with Grade 1 embryos, but pregnancy rates are adequate if embryos are transferred as fresh into suitable recipients. Therefore these embryos are often called “transferable” but not “freezable”.
- Code 3: Poor. These embryos have major irregularities in shape of the embryonic mass or in size, color, and density of individual cells. At least 25% of embryo mass must be intact. These embryos do not survive the freezing/thawing procedure and pregnancy rates are lower than those obtained with fair quality embryos if transferred fresh into suitable recipients.
- Code 4: Dead or degenerating. These could be embryos, oocytes or 1-cell embryos. They are non-viable and should be discarded.

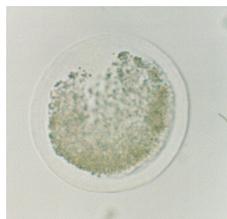
The IETS Manual also states that visual evaluation of embryos is a subjective evaluation of a biological system and is not an exact science. Therefore, pregnancy rates may sometimes be lower than expected due to other factors such as environmental conditions, recipient quality, and technician capability. Generally, unless otherwise agreed, only Code 1 embryos should be utilized in international commerce. Examples of embryos of different stages and quality are indicated in Fig. 1 and 2.

In the superovulated cow, there is likely to be a considerable range of stages of development on any given day after estrus (Mapletoft, 1986). On day 7 after estrus, there may be morula and hatching blastocysts within the same flush. At the same time, there may be embryos of excellent quality and also unfertilized and degenerate embryos. Generally, wide variations in embryo quality and stages of development are signals that the existing embryos are not entirely normal and that pregnancy rates may be disappointing (Mapletoft, 1986). Embryos of excellent and good quality, at the developmental stages of compact morula to blastocyst yield the highest pregnancy rates (Hasler *et al.*, 1987). Other studies have evaluated pregnancy rates according to the stage of embryo development after freezing and thawing. In one study that evaluated 5,287 transfers of embryos cryopreserved in glycerol, pregnancy did not differ between morulae, early blastocysts, blastocysts, and expanded blastocysts (Hasler, 2001). Conversely, other studies found a decrease in pregnancy rates with more developed embryos (blastocysts and expanded

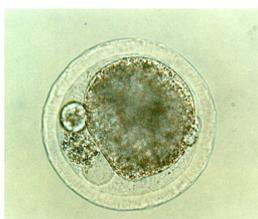


blastocysts; Dochi *et al.*, 1998; Palma *et al.*, 1998; Caccia, 2003; Bó *et al.*, 2012), but in these studies embryos were cryopreserved in ethylene-glycol prior to transfer. Fair and poor quality embryos yield poor pregnancy rates after freezing. It is advisable to select the stage of the embryo

for the synchrony of the recipient. It would also seem that fair and poor quality embryos are most likely to survive transfer if they are placed in the most synchronous recipients (Hasler *et al.*, 1987).



Cycle Day: 7  
Stage Code: 1  
Quality Code: 4



Cycle Day: 7  
Stage Code: 1  
Quality Code: 4



Cycle Day: 7  
Stage Code: 1  
Quality Code: 4



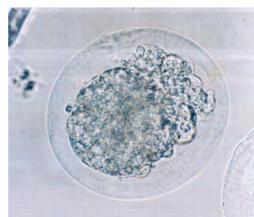
Cycle Day: 7  
Stage Code: 2  
Quality Code: 4



Cycle Day: 7  
Stage Code: 4  
Quality Code: 1



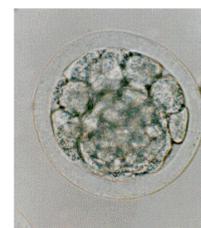
Cycle Day: 7  
Stage Code: 4  
Quality Code: 2



Cycle Day: 7  
Stage Code: 4  
Quality Code: 2



Cycle Day: 7  
Stage Code: 4  
Quality Code: 3



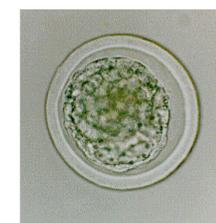
Cycle Day: 7  
Stage Code: 4  
Quality Code: 3



Cycle Day: 7  
Stage Code: 4  
Quality Code: 3



Cycle Day: 7  
Stage Code: 4  
Quality Code: 3

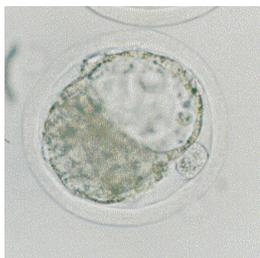


Cycle Day: 7  
Stage Code: 5  
Quality Code: 1

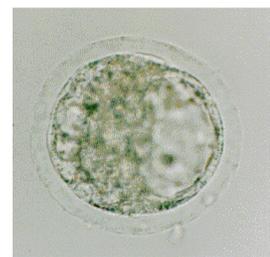
Figure 1. Bovine embryos: examples of developmental stage and quality. Stages 1 to 5.



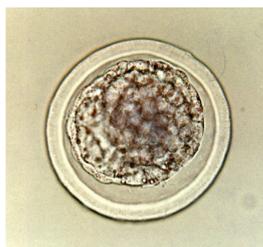
Cycle Day: 7  
Stage Code: 5  
Quality Code: 2



Cycle Day: 7  
Stage Code: 5  
Quality Code: 1



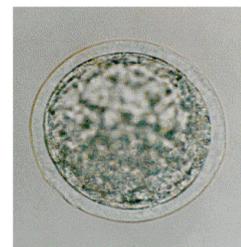
Cycle Day: 7  
Stage Code: 5  
Quality Code: 2



Cycle Day: 7.5  
Stage Code: 5  
Quality Code: 1



Cycle Day: 7.5  
Stage Code: 6  
Quality Code: 1



Cycle Day: 7.5  
Stage Code: 6  
Quality Code: 1



Cycle Day: 7,5  
Stage Code: 7  
Quality Code: 1



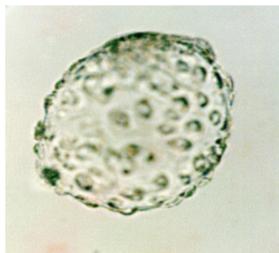
Cycle Day: 7,5  
Stage Code: 7  
Quality Code: 2



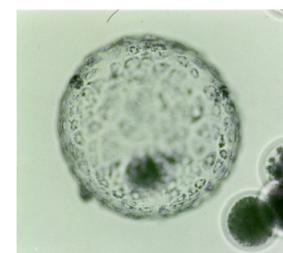
Cycle Day: 7,5  
Stage Code: 7  
Quality Code: 2



Cycle Day: 8  
Stage Code: 8  
Quality Code: 1



Cycle Day: 8  
Stage Code: 8  
Quality Code: 1



Cycle Day: 9  
Stage Code: 9  
Quality Code: 1

Figure 2. Bovine embryos: examples of developmental stage and quality. Stages 5 to 9.



### Summary and final comments

Embryo evaluation is one of the most critical steps of the embryo transfer procedure. The IETS Manual states that embryos must be graded based on a 1 to 9 point system to determine the stage of development and a 1 to 4 system to determine embryo quality. Grade 1 embryos survive well to the freezing/thawing procedure and are recommended for international trade; whereas Grade 2 and 3 must be transferred fresh into suitable recipients. Therefore, the decision as to whether an embryo is worthy of transfer or freezing and whether the embryo is eligible for export will rely on the expertise and experience of the person that evaluates the embryos.

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## Use of bovine sex sorted sperm on timed artificial insemination, *in vivo* and *in vitro* embryo production programs

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**Keywords:** *in vitro* embryo production, sexed semen, timed artificial insemination.

The use of sex-sorted sperm in beef and dairy cattle increases the production of specific gender born calves, increasing the genetic gain progress and efficiency of beef and dairy production. The sex-sorted sperm has been routinely used in various commercial biotechnology such as artificial insemination (AI) upon estrus detection, timed artificial insemination (TAI) and *in vivo* (SOV) and *in vitro* (IVP) embryo production. Currently, it is possible to obtain acceptable conception rates (around 80% of those obtained with conventional semen) after AI with sexed semen in heifers inseminated upon estrus detection. The time of insemination in relation to the onset of estrus and/or ovulation can increase pregnancy outcomes. Greater conception rates is obtained when AI are performed 16 to 24 hours after onset of estrus (i.e. 6 to 14 hours before ovulation). In TAI programs, greater conception rate could be achieved after if the TAI is performed 10 hours before synchronized ovulation. In superstimulated cows, the use of sex-sorted sperm reduces the production of viable embryos, however the delay of 6 hours in the TAI can also increase the number of embryos collected in both *Bos indicus* and *Bos taurus* donors. Currently, IVP technique has been employed to optimize the use of sexed semen. With a single dose of sex-sorted sperm could fertilize approximately 100 oocytes with satisfactory *in vitro* embryo production. However, it is important to note that, regardless of biotechnologies, there is great individual variation on fertility among bulls subjected to the sorting process. These differences should be considered when the sex-sorted sperm is used for AI and embryo production programs.



## Animal cloning, transgenesis and stem cell biology: powerful biotechnological tools for the good of mankind

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**Keywords:** animal cloning, ruminants, stem cell biology, transgenesis.

For the past three decades, the use of animal cloning by nuclear transfer, the production of transgenic animals by genetic engineering (GE), and the outcome and expansion in knowledge in stem cell biology have been envisioned as important biotechnological strategies to boost food quality, animal yield, and for the production of a wide range of bioproducts and procedures that can be used for the benefit of human and animal health. In animals, cloning by somatic cell nuclear transfer (SCNT) has been of importance for research and development, being routinely used worldwide for scientific, conservational, and/or commercial purposes, contributing to advances in many related fields, including epigenetics, genetic reprogramming, developmental biology, and even neonatology. Several applications have been associated with cloning by SCNT, as reproductive cloning, for the genetic conservation and propagation of economically important individuals and endangered species, genetic engineering and transgenesis, stem cell biology, and therapeutic cloning, which may eventually have a direct impact on human health. Next, through transgenesis, GE animals can be used to improve production traits (e.g., AquaAdvantage<sup>®</sup> salmon, AquaBounty), to reduce or minimize the impact of animal production on the environment (e.g., Enviropig<sup>™</sup>, University of Guelph), to add value to an animal product (e.g., human lysozyme goat milk, University of California at Davis and University of Fortaleza; human lactoferrin cow milk, Pharming Inc.), to promote disease resistance (e.g., chicken that do not transmit bird flu, Roslin Institute), as animal models for biomedical applications (e.g., transgenic goat models for cardiac fibrosis, Utah State University), for entertainment as *bio-art* (e.g., GloFish<sup>®</sup> fluorescent fish, Yorktown Technologies), and to produce recombinant proteins in blood, urine, semen, salivary gland, egg white or milk that can be collected, purified and used as pharmaceutical or biosimilar products (*biopharming*) or even for industrial and general use (e.g., Spider Silk transgenic goats, Nexia-Utah State University). On its turn, stem cells have been of great interest, hence being widely studied for their plasticity and potential therapeutic use. Mouse embryonic stem cells (ESC) have been extensively used both as a model for the study of cell lineage and regulation of gene expression during mammalian development and as a vehicle for genetic engineering, functional genomics, and cell therapy studies. Animal and human ESC promise to offer similar uses as in the mouse, in addition to new opportunities for use in study of early embryo development, creation of disease models, regenerative medicine, and cell and gene therapy. However, proven *bona fide* ESCs are yet to be obtained in livestock species. In addition to ESC, stem cells from fetal or adult somatic (mesenchymal, dental pulp, etc.) and germline (GC) origin, amniotic and cord blood (CB-SC) stem cells, and more recently, the induced pluripotent stem cells (iPS), have demonstrated functional potency and self-renewal capacities, with great potential to be used as tools for *in vitro* differentiation and/or cell therapy for tissue remodeling, regeneration and engineering. In fact, adult stem cells have been routinely used in medical therapies, as for example, in bone marrow transplantation used to treat leukemia in humans. In summary, cloning by SCNT, transgenesis by GE, and stem cell research have been shown as robust and viable technological strategies to aid in the resolution of problems of the modern world. However, there still exists a great deal of social, ethical, religious and scientific uncertainties surrounding research and applications in such fields. Despite some skepticism by the general public, the use of such technologies has already been translated in the development of new drugs, procedures and even therapies to the benefit of animals and humans. Some of such recent developments, potential applications, and novel understanding in biological processes associated with cloning, transgenesis, and stem cell technologies will be presented and discussed by distinguished speakers at the II Symposium of the South American Research Consortium on Cloning and Transgenesis in Ruminants.



## **Lentiviral-vectors as an efficient screening method to evaluate transgene expression in livestock**

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**Keywords:** cattle, lentiviral vectors, transgenic animals.

Several methods including microinjection, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), nuclear transfer, sperm-mediated gene transfer and germ cell transfer afford relatively efficient routes to transfer transgenes to the germ line of transgenic livestock. Founder animals generated by these technologies are typically bred to produce the desired populations. Alternatively, there are certain circumstances where the transgenic studies may not need breeding populations but instead quick evaluation of gene expression or gene re-regulation/deregulation. In those circumstances, production of transgenic animals using lentiviral-mediated transgenesis is an attractive option. Lentiviruses are members of the extensive family of complex retroviruses. Lentiviral vectors have a large payload capacity for carrying DNA into cells. Typically, the DNA size is about 8-10kb, which is the DNA construct size needed for most transgenic applications. Furthermore, lentiviruses can be produced at high titers, which can increase the efficiency of transgenesis. This technology has been used to produce transgenic mice, rats, cats, swine, sheep, goats, cows and non-human primates. One advantage of lentivirus-based vectors is that the expression of the transgenes are typically sustained and not silenced as occurs in many virus-produced transgenic animals. Furthermore, transgene expression from lentiviral-transferred genes is often reflective of the transgene copy number that has integrated into the genome. The ability to analyze the relationship between transgene expression level and the phenotype in founder animals directly could represent significant cost savings for conducting transgenic studies. Recently, we have been developing lentiviral-based vectors to target proteins into the mammary gland of cattle. We have been using mammary epithelial cell lines (murine and bovine) to evaluate transgene expression before producing transgenic animals. Our lab is ultimately interested in developing the bioreactor potential of the bovine mammary gland for human and animal nutraceutical and pharmaceutical production.



## **Insights on bovine genetic engineering and cloning**

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**Keywords:** bovine, induced pluripotency, somatic cell nuclear transfer, transgenic technology.

Transgenic technology has become an essential tool for the development of animal biotechnologies, and animal cloning through somatic cell nuclear transfer (SCNT) enabled the generation of genetically modified animals utilizing previously modified and selected cell lineages as nuclei donors, assuring therefore the generation of homogeneous herds expressing the desired modification. The present study aimed to discuss the use of SCNT as an important methodology for the production of transgenic herds, and also some recent insights on genetic modification of nuclei donors and possible effects of gene induction of pluripotency on SCNT.



## **The prevention and treatment of diarrheal illnesses using the milk from transgenic animals**

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**Keywords:** diarrhea, goat, lysozyme, lactoferrin, transgenic animals.

Human milk contains the antimicrobial factors lysozyme and lactoferrin that help contribute to the development of a healthy intestinal tract. These key factors are lacking in the milk of common dairy animals such as the cow and goat. Transgenic goats expressing human lysozyme in their milk and transgenic cows expressing human lactoferrin in their milk are being studied as sources of milk that could help fight the diarrheal illnesses that claim the lives of more than 1.5 million children worldwide under the age of five each year. This work has progressed over the past 20 years from testing hypothesis of using transgenics to modify the properties of milk in a mouse model to the testing of the efficacy of the milk to act at the level of the intestine in a novel pig model of human health. All work to date has indicated that the milk from lysozyme transgenic goats and lactoferrin transgenic cows positively impacts gut microbiota and morphology, can mitigate intestinal damage caused by malnutrition and helps to resolve *E. coli*-induced diarrhea more quickly than control milk. Work is now focusing on the mechanism of action of these antimicrobials and moving to the rat for toxicity testing as one of the last steps before human clinical trials.



## Development of transgenic goat models of cardiac fibrosis

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**Keywords:** animal cloning, animal model, goat, transgenic animals.

Fibrosis is a fundamental element of the damaging structural remodeling of cardiac tissue seen in a wide range of cardiac disease. In fibrosis, profibrotic factors act on cardiac cells to increase deposition of extracellular matrix. These changes alter the structure, architecture and shape of the heart affecting three cardiac functions: ventricular contractility, valvular performance and electrical conduction. Transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) has a potent profibrotic function and is central to signaling cascades involved in interstitial fibrosis. We recently developed an efficient procedure for generation of transgenic goats using somatic cell nuclear transfer (Hall *et al.*, *Reprod Fertil Dev*, 2012; 25:162) and produced cloned transgenic goats expressing human TGF- $\beta$ 1 under control of the cardiac-specific  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) promoter. Cardiac biopsies were obtained using a bioptome under visualization of intra-cardiac echocardiography and cardiac specific expression of hTGF- $\beta$ 1 was confirmed. Current status of the model characterization will be reviewed. Additionally, the presentation will discuss strategies for early non-invasive *in vivo* detection of gene expression during large animal model development and approaches for achieving reproducible gene and protein expression levels.

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## Uses of mesenchymal stem cells in tissue engineered regeneration

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**Keywords:** animal model, pig, stem cell biology, tissue engineering.

Bone is a living polymer with substantial healing capacity. However, extensive bone loss due to disease or trauma may require tissue-engineering methods. Presently, autologous bone grafting is the gold standard for bone repair, but presents limitations including donor site morbidity, bone shape, and amount. Synthetic bone grafts such as ceramics, collagen, non-collagenous proteins, and biodegradable polymers that have been tested also have drawbacks related to poor resorbability, use of processed animal components, inferior handling characteristics, and cost. The use of stem cells appears a means to overcome such limitations. Bone marrow mesenchymal stem cells (BMSC) have been the choice, to date, for stem cell therapy for bone regeneration. Adipose-derived stem cells (ASC) are more abundant and accessible with lower donor site morbidity, making them a potentially better alternative to BMSC. Once ASC are obtained, it is critical to establish a proper animal model that closely resembles the size of human bones for their use in pre-clinical trials. Among available animal models, swine are the closest non-primate model for craniofacial configuration with two dentitions. Application of stem cells for regeneration of clinically relevant defects will require scaffolds that provide a nurturing environment, temporary function, replicate complex anatomic defects while being readily fixed to surrounding bone and be surgically implantable. The porcine animal model provides a valuable tool for scaffold use in tissue engineered bone regeneration with the use of ASC especially for complex anatomic defects in the craniofacial region.



## Evaluation of risks from environmental contact with transgenic livestock

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**Keywords:** livestock, risk assessment, transgenic animals.

Assessment of general risk posed from transgenic animals is important to their future contributions to society. Identification of potentially harmful properties of transgenic livestock is the initial step in a risk assessment. We previously developed and characterized transgenic swine containing a mammary-specific transgene, bovine  $\alpha$ -lactalbumin, (B $\alpha$ -LA) that results in increased milk production in sows. This study determined if B $\alpha$ -LA is expressed in tissues of transgenic swine (T) other than the lactating mammary gland and if the transgene DNA (Tg) crosses into non-transgenic swine under various physiological and physical conditions. Specific aims were to determine whether Tg can be (1) expressed in tissues other than the mammary gland of a T sow; (2) transferred to non-T swine by direct physical association or contact; (3) transferred to non-T swine via mating; (4) transferred to non-T swine during gestation, parturition, or lactation. To address specific aim 1, T and non-T (control; C) pigs were raised to 180, 220, 250 days of age, or 112 days post-breeding and then sacrificed for tissue collection. For specific aim 2, comparable age- and weight-matched T and C pigs were housed together to allow for general contact that is normal in swine production, starting from weaning (21 days) to either 180, 220, or 250 days of age and then sacrificed for tissue collection. In experiments 1 & 2, blood, brain, jejunum, kidney, liver, lung, mammary gland, muscle, ovary, sublingual salivary gland, skin, and spleen were collected. For specific aim 3, vaginal, cervical, uterine, oviductal, and ovarian tissues were collected from C females at 2, 7, or 90 days post-mating to T males, and penis, bulbourethral gland, urethra, testis, and epididymal tissue was collected from C males 7 days after mating to Tg females. Addressing specific aim 4 was divided into 3 sub-experiments: 1) tissues from 112 day fetuses were collected from C sows bred to a C boar and T sows bred to a C boar; 2) C piglets were removed from their birth dam at parturition and before they had suckled, fostered to a lactating T sow or to a lactating C sow, and allowed to suckle for 24 or 72 h before sacrifice and tissues collection; 3) C piglets were allowed to suckle their birth dam until 3 days of age, then fostered to a lactating T sow or to a lactating C sow, and allowed to suckle for 72 or 168 h before sacrifice and tissues collection. Jejunum, liver, lung, muscle, and skin was harvested for each sub-experiment. The presence of the Tg or its expression in tissues from C and T animals was tested by PCR analyses. In total, 1,626 tissues from 295 animals were analyzed. The Tg was not expressed in tissues other than the mammary gland of a T lactating sows. The Tg was not detectable in any tissue sample C animals after co-habitation for 180, 220, or 250 days or at 2, 7, 90, or 112 days post-mating. At day 112 of gestation, all samples from C piglets whose dam was a Tg female were negative, except for the outer placental membrane, which screened positive for Tg. The latter tissue is derived from the maternal tissues and would be expected to be positive for Tg in a Tg female. Tissues from C piglets cross-fostered prior to suckling (day 0) or 3 days after birth to a lactating T sow were negative for Tg. These results strongly indicate that horizontal Tg transmission between T and C pigs does not occur during co-habitation, mating, gestation, or lactation.



## **Whole animal assessment of unintended effects of foreign gene products on host and non-target organisms**

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**Keywords:** goat, human lysozyme, milk, risk assessment, transgenic animals.

Risk assessment is an important aspect for the future use of transgenic animals. While much work has been carried out determining the risks of transgenic plants and their interactions with their environments, risk analysis in transgenic animals is less defined and different parameters need to be assessed. The first goal is risk assessment in animals is to ensure that animal health and welfare are not compromised by the presence and expression of the transgene. Next, the intended function of transgene product should be verified followed by other context-specific types of analyses to determine any associated risks the transgene product may have on both the animal and consumers of the product produced from the transgenic animal. Work can also be done to assess any unintended consequences of transgene expression at the whole animal level by conducting global types of analyses to determine if a transgene product can impact aspects of an animals' physiology other than those intended by the function of the particular transgene. The Artemis line of transgenic goats that expresses the antimicrobial human lysozyme in their milk was established in 1999 and has been subjected to multiple risk assessment characterizations. The growth, reproduction and milk production of the transgenic line is in no way adversely impacted by the presence or expression of the lysozyme transgene. Unintended effects of the transgene have also been assessed using microbial and metabolite profiling techniques to investigate any effects on the physiology of the host (lactating goats) and non-target organisms (kid goats consuming the milk). The microbial profile of lactating does changed more over time than it did in response to expression of the transgene as did the metabolite profiles of kid goats consuming the milk. These types of global analyses were useful in assessing the scope of pleiotropic effects of transgenes and their products at the whole animal level. Data collected on the intended and unintended effects of transgenes is an important part of the development of a transgenic line and will contribute to the ability to make science-based decisions about the safety and future implementation of transgenic food animals.



## The endometrium of cows as a source of mesenchymal stem cells

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**Keywords:** cattle, cell therapy, gene expression, stem cell biology.

Adult mesenchymal stem cells had been isolated from numerous tissues of different animal species; however endometrial stem cells, only from human, mice and recently from pigs, but not from cattle. It has been reported that human endometrial stem cells are more plastic than adipose or bone marrow derived (Bockeria et al., J Transl Med. 2013 Mar 5;11:56. doi: 10.1186/1479-5876-11-56). The aim of our work was to identify such cells in the bovine endometrium and to establish a model system in which to test inducers of differentiation and recruiters of stem cell niches, for potential therapeutic use in other species, such as horses. We searched for endometrial stem cells in healthy cycling cows and in cattle with clinical (C) or subclinical (SC) endometritis. For this, the uterine tracts of slaughtered cows were collected at early (days 2-5; ELF) and late luteal phases (days 11-15; LLF) of the estrus cycle of healthy cows. For endometritis diseased cattle, uterine biopsies were taken in live animals. In all cases, markers of stemness, inflammation, uterine function and housekeeping were studied both at mRNA and protein level, by RT-qPCR and Western blot/immunohistochemistry respectively. In addition, cell primary cultures were established in vitro from all the animals (n=4 for ELF, n=4 for LLF; n=4 for C and n=4 for SC). We found that the endometrium of most animals expressed embryonic stem cell markers, such as OCT4 and SOX2, but not or little NANOG, as well as CD44, c-Kit and STAT3, markers of mesenchymal stem cells. The expression profile of these markers, was not related to the stage of the estrus cycle; however there was a statistically significant reduction in the expression of embryonic stem cell markers in ill animals, being the lowest in clinically ill and intermediate in subclinical endometritis, ( $P<0.05$  and Pearson's correlation coefficient 0.92). For markers of multipotency (mesenchymal), the expression was lower in clinical endometritis ( $P<0.05$ ). In resume, the expression profile of stem cell markers is indicative of the presence of stem cells in the bovine endometrium. At the protein level, we verified our findings for OCT4, SOX2 and CD44 using Western blot and immunohistochemistry. In general there was a concordance between mRNA and protein profiles. Inflammatory markers showed a pattern characteristic for each of the stages studied. In order to have an ultimate criterion of the presence of stem cells, we tested the differentiation potential of the isolated cell lines, upon induction to chondrogenic, osteogenic and adipogenic lineages. We found that the entire cell lines tested (n=8) displayed mesenchymal differentiation potential as demonstrated from specific stainings as well as expression of gene markers. At present, work is in progress to isolate pure stem cell populations from these primary cultures to have a deeper characterization of the cells. We showed for the first time, the presence and differentiation potential of endometrial stem cells in cattle, this can have impact on the development of new therapeutic approaches to combat uterine diseases, such as endometritis or endometrosis (in horses).

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## Genetic modification of bovine embryos by lentiviral vectors

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**Keywords:** cattle, embryo, lentiviral vectors, transgenic animals.

Lentiviral vectors have been widely used in studies for generation of human induced pluripotent stem cells (Okita and Yamanaka, 2011. *Philos Trans R Soc Lond B Biol Sci* 366:2198) and for human gene therapy (Antoniou et al, 2013. *Hum Gene Ther* 24:363). Such vectors have also been shown to be an alternative to generate livestock. As any other transgene delivery systems, lentiviral vectors have pros and cons. Its efficiency is elevated when compared to other systems. Lilico et al. (*Trans Res* 20:441, 2011) generated more transgenic lambs by lentiviral vectors in 2008/2009 (32 founders with 6 different transgenes) than the previous 25 years in the Roslin Institute using other techniques. The efficiency of lentiviral vector seems to be related to its nuclear import feature and ability to integrate into the genome of non-dividing cells (Durand and Cimareli, 2011. *Viruses* 3:132). However, the production and manipulation of these vectors require laboratories with biosafety level two, despite the third generation of lentiviral vectors has features that increases the biosafety and reduces undesirable effects as those caused by retrovirus, as activation of proto-oncogenes (Cockrell and Kafri, 2007. *Mol Biotechnol* 36:184). The transfer vector size, generally smaller than 13 kb, can be a limitation, allowing inserts with up to 7.5 kb only (Al Yacoub et al., 2007. *J Gene Med* 9:579). Moreover, expression of lentivirus integrants may be modulated by epigenetic modification and disturbs transgene expression (Hofmann et al., 2006. *Mol Therap* 13:59). The usefulness of lentiviral vectors to generate transgenic cattle was reported by Hofmann et al. (*Biol Reprod* 71:405, 2004) by microinjecting lentiviral particles into perivitelline space of matured oocytes. Microinjection into perivitelline space of bovine zygotes was shown to be less efficient than of oocytes (Hofmann et al, 2004. *Biol Reprod* 71:405; Ewerling et al., 2006 *Transgenic Res* 15:447). We have also carried out studies with lentiviral vectors to delivery GFP transgene to matured bovine oocytes and zygotes. Differently from previous studies, we microinjected lentiviral particles into the perivitelline space of zygotes with 6h post in vitro fertilization in an attempt to make the transgene available before syngamy. Fifty percent of the blastocysts produced had the transgene detected by PCR in contrast to 100% of blastocysts produced from matured oocytes microinjected with lentiviral vectors. In both groups, the proportion of blastocysts emitting green fluorescence was lower than that of blastocyst with the transgene detected by PCR, suggesting the silencing of GFP expression in some embryos. Eleven blastocysts produced from matured oocytes microinjected with lentiviral vectors were transferred to synchronized recipients and resulted in five pregnancies (45.4%); rate similar to that regularly reported with non-microinjected vitro-fertilized embryos. However, one fetus was lost in the 8th month of pregnancy and two out four calves died few hours before parturition without any apparent morphological alteration. The transgene was detected by PCR in umbilical cord and blood cells from one of the stillborn calves while tissues from other three calves are still under evaluations. Those results indicate that the use of lentiviral vectors by microinjection into perivitelline space of bovine oocytes and zygotes still demands improvements. Nevertheless, lentiviral vectors can also be used to transduce somatic donor cells in order to generate transgenic cloned animals and may be an alternative for production of transgenic cattle (Monzani et al. 2013. *Gen Mol Res* 12). Despite its potential application for cattle transgenesis, lentiviral vectors may become restrict to production of transgenic cows for secretion of recombinant biopharmaceutical proteins for human and animal health purposes. As the current lentiviral vectors are based on HIV-1 nucleotide sequences, the consumer may decline to consume milk or meat produced by cattle genetically modified by those vectors. Besides, new tools to edit the genome, as meganucleases, are becoming available for livestock and may have advantages over lentiviral vectors.

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## **Back to the future: embryo microinjection and Meganucleases**

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**Keywords:** embryo microinjection, meganucleases, transgenic animals.

The production of transgenic livestock between the early 1980s and the late 1990s was principally based on the technique of pronuclear microinjection, although there was some production of transgenic livestock using retroviral vector-mediated and sperm-mediated transgenesis. Pronuclear microinjection was a reliable method to produce transgenic livestock, but the technique was labor-intensive, required a reasonable amount of skill, and was limited to the random introduction of DNA sequences. With the birth of Dolly in the late 1990s, the production of transgenic livestock shifted to the use of cells genetically engineered in culture followed by somatic cell nuclear transfer-based cloning to generate transgenic animals. SCNT-based cloning has the advantage of allowing for gene targeting, and thus the production of knockouts, but still suffered from being technically demanding and low efficiencies of obtaining live born clones. While SCNT-based cloning was being developed for the production of transgenic animals other avenues were also being explored. Two of the most successful were lentiviral, instead of retroviral, vectors and transposons adapted for use in vertebrate cells. Both approaches were successful and have the advantage of high efficiency of gene transfer, but both still suffer from only being able to add genes and cannot be used for targeting. Lentiviral vectors also have the disadvantage of only being able to carry a small construct, as the amount of DNA that you can insert into the viral capsid is limited. The most recent developments affecting the production of transgenic animals is the development of the designer meganucleases. These include zinc finger nucleases (ZFN), TALENs, and most recently the CRISPR-Cas system. In each case the enzyme system can be targeted to cut at a specified location in the genome, thus providing a mechanism for gene targeting. Meganucleases and the accompanying DNA for integration can be injected into an embryo and achieve a high success of gene targeting, so we have come full circle from the initial techniques of pronuclear microinjection back to embryo injection with gene targeting potential.



## Deciphering gene expression profile of early bovine embryos: insight for successful development

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**Keywords:** animal cloning, cattle, embryo, gene expression.

It is well known that embryos produced *in vitro* are less competent than their *in vivo*-derived counterparts. When embryos are produced or manipulated *in vitro* their developmental potential decreases significantly what impinges on the production of viable offspring. The efficiency and the final outcome is proportional to the complexity of these technologies, being somatic cell nucleus transfer (SCNT) the most complex and inefficient, giving no more than 10% of successful pregnancies. The lower quality of *in vitro* produced embryos is due to changes in their gene expression pattern as a result of the interaction with the adverse environment generated by the *in vitro* conditions. When embryos are produced by SCNT this scenario is even more critical since the cloned embryo is derived from the transfer of a somatic cell into an enucleated oocyte, which is responsible for reprogramming the differentiated nucleus in order to induce gene expression patterns compatible with embryonic development. More often the reprogramming process is not capable to establish an appropriate gene expression pattern what leads to a low competent embryo that probably will not develop to term. It had also been seen that early embryo morphology does not correlate with an appropriate gene expression pattern, making difficult embryo selection. It seems that ideal method for embryo selection would be based on the screening of gene markers that correlate with successful pregnancy after embryo transfer. In that sense we have proposed a method to select competent embryos based on the expression of crucial genes at blastocyst stage. Based on the literature and in our own experience we hypothesize that the expression of pluripotency markers (Oct4, Sox2 and Nanog) at blastocyst stage is predictive of *in vivo* bovine embryo development. For that we characterised gene expression pattern of early (Day 7) bovine cloned and IVF blastocysts, with emphasis in the pluripotency markers and correlated this gene expression with embryo quality at blastocyst stage (Day 7). Firstly we found that higher percentage of development to blastocysts in culture correlates with the highest gene expression level of pluripotency markers (Oct4, Sox2, Nanog, Fgf4 and Cdx2;  $P < 0.05$ ) for both IVF and cloned embryos. This correlates as well with a higher total cell number in the blastocysts and might be of practical use for the selection of cell lines for cloning; those cells that yield higher percentage of blastocysts would probably produce more competent cloned embryos. However, we observed that embryos with a highest expression of pluripotency markers also showed greater variability of expression of these genes, suggesting that only few of them underwent a normal reprogramming process. In mouse, Oct4, Sox2 and Nanog are crucial for normal embryo development by controlling early cell fate; also Oct4 will keep an open chromatin in early embryos what is highly correlated with developmental potential of these embryos. If the same would be true for bovine embryo development, then it can be expected that high expresser embryos will produce greater successful pregnancies; however at least in our hands, the final outcome (10% of live offspring) was not improved by using cell lines that yield greater blastocyst percentage (65%) and highest level of pluripotency genes expression. In order to find out if the portrait of gene expression at Day 7 influences gene expression at elongation (Day 17; filamentous stage), we split Day 7 embryos (IVP) and transferred one half to a temporary recipient cattle, while the other half was used to analyze gene expression of several developmentally important genes. Firstly we optimize the splitting method in order to have similar hemi-embryos. More than 65 % of the split embryos generated two viable hemi-embryos with the same ability of *in vitro* re-expansion, similar cell number as well as homogenous gene expression (Oct4, Sox2, Nanog, Cdx2, Bcl2-l1 and BAX). Transferred hemi-embryos were recovered at day 17, classified by the elongation stage and used for gene expression analysis of the mentioned genes. From 15 embryos that were transferred, 9 (60 %) were collected with different grades of elongation (1-15 cm). A correlation analysis showed that the expression level of pluripotency markers (Oct4, Sox2 and Nanog) at blastocysts correlated with the expression level of the same genes at the elongation stage, but neither with the expression of the two trophoblastic markers analysed in the elongated embryos (Cdx2 and TP1), nor with embryo length. There are evidences suggesting a different from canonical (as for mouse and humans) role of pluripotency markers in bovine embryo development. From our work, we concluded that at least expression of Oct4 is not a good marker to predict bovine embryo quality. Functional analyses are necessary to define the actual role of the pluripotency genes in bovine embryos and their relation with early development.

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## **Interface between scientific and commercial applications on cloning, transgenesis and stem cell biology: A South American perspective**

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**Keywords:** animal cloning, animal transgenics, stem cell biology.

The interaction of academia and industry is one of the major aspects related to the success of technology generation and dissemination along the productive sector. In the industrialized countries there is a very important issue related to this interaction and it is reverted in research financing. South American countries relies however to a mostly governmental investments on research and technology. This late information is however changing lately due to modifications in the research agencies and government politics. Agriculture production in South America increased the productivity in dramatic proportions within the last 5 decades, leading to be recognized as one of the major players of the food production and exportation in the world. The efficiency on agricultural production shed light to many other possibilities on the agriculture including the production of fuel and lately energy and finally ended up by increasing the price of the agricultural products with benefits the producers. In this scenario farmers were trained to believe in the technology and to rely on it to increase the productivity. In other hand, the beef cattle producers were somehow challenged by a highly efficient agriculture to increase the productivity or to move to rare new agricultural frontiers. Altogether, this led to an increase of the value of the bulls and semen, and especially to the genetic selection. The investment in genetics selection by cattle breeders reached a point where they needed to produce “elite” animals in order to offer to the market solutions to increase productivity. ET and then IVF came as the first and second wave of technologies related to this needs. Both, but specially IVF due to its capability to scale up the elite animals productions were very well accepted in the field and somehow responded to the breeders demand. The nuclear transfer cloning found somehow a window of opportunity on this issue, leading the IVF labs to invest on this technology. Nowadays the production of embryos are reaching a large scale proportion following the FTAI, throughout the FTET, using sexed semen, non additive genetics and indicating a great potential to produce the future. What about clones, transgenic and stem cells biology? They are part of the new coming technologies. Due to the long generation intervals, lineages as observed in pigs and chicken are not available in cattle. Animals produced by large scale cloning technologies, possibly resulted from stem cells technologies, will in a certain moment reach the ground and change the cattle production system. There are already investments in research by public and private sector with this aim. Together, in the next decades, this technologies and transgenic modification will change a paradigm of cattle production in South America and other regions in the world.



## **Interface between scientific and commercial applications on cloning, transgenesis and stem cell biology: a North American perspective**

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**Keywords:** animal cloning, animal transgenics, stem cell biology.

Somatic cell nuclear transfer (SCNT), or cloning, is one of the assisted reproductive technologies (ARTs) currently used in agriculture. ARTs have a very long history, for example artificial insemination has been employed for several hundred years, and some others (embryo transfer, IVF, embryo freezing) have been extensively used in livestock breeding for decades. SCNT does not require fertilization and therefore it allows for the propagation of proven genotypes without “genetic reshuffling”. Commercial applications in agriculture involve employing SCNT for the expansion of elite genetics, the propagation of lost genetics and the protection of genetics in the event of a catastrophe such as foot and mouth disease. The benefit of cloning high-quality individuals will be to increase the number of descendants of elite genotypes in the breeding population via enhanced and prolonged production of a large number of offspring. Previously population outliers (especially maternal lines) had insignificant impact on the population mean. SCNT can amplify the impact of unique genotypes on the population. The current status of SCNT utilization for agricultural and biomedical applications will be reviewed including cloning efficiencies in various livestock species and reproductive performance of clones. Regulation and development of animal biotechnologies in the U.S. will also be discussed.



## **Interface between scientific and commercial applications on cloning, transgenesis and stem cell biology: a dairy industry perspective**

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**Keywords:** animal cloning, animal transgenesis, cattle, dairy industry.

Dairy cattle production was transformed by the advent of artificial insemination because bulls could be selected for desirable traits very accurately and their genetics disseminated widely. The advent of superovulation and procedures for in vitro production of embryos did not have a major impact on the dairy industry because genetically-superior females could not be identified with much accuracy and the number of offspring produced by females remains much lower than for offspring produced by bulls. Development of genome-wide selection tools such as the Illumina SNP50 and High Density chips has meant that genetically-superior females can be identified with reliabilities that approach that achieved for bulls through progeny testing. It is now possible to use genomic selection to identify genetically superior individuals as early as the preimplantation embryo stage. While genomics has leveled the playing field somewhat for genetic selection on the female vs male side, it remains true that the number of offspring produced by females remains much lower than for offspring produced by males. This situation may be changed, however, by the advent of stem cell technologies that allow differentiation of embryonic or pluripotent stem cells into oocytes. In addition, there is evidence for naturally-occurring oocyte stem cells. If real and if systems for culturing such cells can be developed, it could be possible to generate unlimited numbers of offspring from genetically-superior females. The advent of gene editing technologies, for example using zinc finger nucleases or TALENS, means that embryos produced in vitro could be generated with specific mutations in economically-important traits. Taken together, the development of new technologies means that the dairy industry is poised for advances in genetic selection on the female side that could rival that achieved by artificial insemination.



## ***In vitro* embryo production by Ovum Pick Up and ICSI in the horse**

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**Keywords:** equine, fertilization, oocyte.

In vitro embryo production in livestock, especially in bovine, has been developed to a very advanced and reproducible stage, to the point that it replaces in many instances conventional multiple ovulation and embryo transfer. In contrast, the pace of technological progress in equine has been much slower, due to the anatomical and physiological limits typical of this species, and to the inefficiency of conventional in vitro fertilization. However, in vitro embryo production associated with oocyte recovery from live donor mares (Ovum Pick Up, OPU) and ICSI (Intracytoplasmic Sperm Injection) is becoming more and more popular and finds its applications in the clinical setting not only for treating female infertility but also male infertility, overcoming limited availability of semen or poor quality semen, and even allowing out of season breeding programs also for sport performing animals, etc. The presence of functional ovaries with growing follicles is the main requirement for OPU, therefore mares of different age and reproductive status are suitable donors. Oocytes can be recovered from preovulatory follicles 24-30h after hCG or GnRH priming of a follicle larger than 35 mm to initiate in vivo maturation, or from all antral follicles present at any given time, larger than 1cm that are then subjected to a full in vitro maturation. Oocytes from preovulatory follicles have been reported to have a higher developmental competence compared to in vitro matured ones however usually there is one follicle at a time for recovery and this procedure can be performed only in cycling animals. On the contrary, collection of immature oocytes can be performed at any time, with some limitation during the deep anestrus, but being very efficient at spring or fall transition due to the presence of many medium sized follicles (1-2 cm). During the breeding season to maximize the number of medium sized follicles careful monitoring of the estrus cycle is required to avoid the dominant follicle and aim at an emerging follicular wave. Recovery rate can be 60 to 70% with an average of 10 oocytes per OPU and the procedure can be repeated at 10-15 days interval with no side effects. Oocytes are matured in vitro for 24 to 28 hours and about 60% reach the metaphase II when they are injected with a sperm. ICSI is performed with a blunt pipette driven by a piezo electric manipulator, this tool greatly facilitate the penetration of the zona pellucida and the rupture of the oolemma to inject the sperm directly into the cytoplasm with minimal damage to the oocyte. Cleavage rate after ICSI is usually in the range of 60 to 70% but with considerable individual differences between donors. After ICSI the injected oocytes are transferred to in vitro culture for 7-8 days until they reach the blastocyst stage. Only about 8% of the matured oocytes develop to the blastocyst stage and can be transferred or frozen for later transfer. After non-surgical transfer of fresh or conventionally slow frozen embryos pregnancy rate is around 60% and pregnancy losses about 20%. Important differences in developmental competence have been observed between breeds: the highest rate in Warmblood and the lowest in Arabians. On average 0.5 embryos are produced for each OPU session, ranging from 0 to 6. This technique offers a solution for treating both female and male infertility and to increase the number of foals from stallions with limited sperm available.



## Metabolic Syndrome and Reproduction: a mystery to be unraveled

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**Keywords:** anovulatory follicles, embryo production, pregnancy.

The terminology Metabolic Syndrome was first suggested in 2002, by Johnson, in an analogy to the human Metabolic Syndrome (Johnson, 2002) and was recently described by the American College of Veterinary Internal Medicine (2010). Animals are obese, present regional adiposity, insulin resistance, hyperinsulinemia, a proinflammatory state and predisposition to laminitis as main characteristics (Frank et al., 2010; Gallantino-Homer; Engiles, 2012; Morresey, 2012). Specifically, laminitis is the most common symptom and in most occasions is the first complaint, and due to its life-threatening aspect, is the biggest concern. The exact physiopathology that explains the alterations detected in the affected animals are still far from being understood, however hormonal, genetic and nutritional aspects are intermingled (Morresey, 2012). Obesity represents an important aspect of the syndrome, as adipose tissue produces a series of adipokines that directly affect insulin metabolism. In Reproduction, several authors suggest an effect of Metabolic Syndrome on cyclicity and pregnancy, though few studies were performed and mechanisms associated with this alterations are not well established. Leptin, a hormone produced by adipocytes signaling to the hypothalamus that there is excess of energy, is normally in high levels in affected animals, maybe cause by a leptin resistant state. There were studies in which cyclicity was evaluated and seasonality was lost in hyperleptinemic individuals (Ferreira-Dias et al., 2005). Vick et al. (2006) found high levels of leptin and insulin in obese animals, along with low levels of thyroxin, reduced insulin sensitivity and estrous cycle alterations, such as increase in the interval between ovulations and longer periods with high progesterone circulating levels. It is important to highlight that several animals are misdiagnosed as hypothyroid due to low levels of thyroid hormones detected, however this characteristic is a consequence of metabolic syndrome and not the cause of alterations. In the other hand, Waller et al. (2006) evaluated high body condition score mares that were hyperleptinemic and no alterations regarding ovarian function and hormonal concentrations were found. When the pregnant status is considered, it is well know that a physiologic insulin resistance occurs in order to supply nutritional support for the fetus and placenta. However, those individuals with a preexisting insulin resistance is present, the condition is exacerbated, leading a worsening of hyperinsulinemia, and systemic, increasing the risks of developing laminitis and loosing the pregnancy (Morresey, 2012). Therefore, when facing an obese animal presenting reproductive problems and laminitis, Metabolic Syndrome should be considered as a possible diagnosis and treated accordingly to avoid worsening of the condition and life threatening situations. This research field requires attention since it has important consequences for the animal health and its reproductive efficiency.

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## Clinical cases of reproductive endocrinopathies in the horse

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**Keywords:** anovulatory follicles, embryo production, pregnancy.

The most common endocrinopathy affecting reproductive performance in mares is PPID (pars pituitary intermedia dysfunction) more commonly known as “Cushing’s “ disease. This disease is difficult to confirm by laboratory testing as many false negatives and false positive may occur. The veterinary practitioner must learn the clinical signs of this disease and use their clinical skills to identify the presence of this endocrinopathy. Laboratory tests will confirm about 70 % of these cases. Most mares are an average of 14 years old when clinically diagnosed with PPID; however, many cases are much younger some are only 4 years of age. The important point here is that PPID takes about 14 years to completely manifest with a full array of clinical signs. Initially only one or two clinical signs may be present such as anovulation or immune suppression. Hirsutism is the most prevalent and consistent sign associated with PPID and it is the first sign to improve when appropriate drug therapy with pergolide and /or cyproheptadine is initiated. Repeated abortion is often a result of depressed immunity caused by PPID. Equine metabolic syndrome is now more completely understood and its detrimental effects on reproductive function are also better known. Simply stated insulin resistance (IR) is the most important component of this disease and can be easily determined by a fasting serum test for Insulin or by use of an oral sugar test to assess the rise in insulin after the sugar challenge. For example, in normal horses Insulin remains below 20 mIU per ml following 15 ml of “Karo” syrup per 100 kg per os. This is a very simple and cheap test to identify IR. Other common uses of endocrine assay are to assess fetal placental function. Especially in cases of placentitis. When placental infection is present the fetus is stressed eliciting an increased secretion of fetal adrenal steroids, which are converted primarily to pregnanes (progestagens) in the peripheral circulation. When clinical signs such as vaginal discharge and or udder engorgement are present in pregnant mares that are 150 days of gestation and beyond, a single serum assay for total estrogens and progestagens can be very useful to assess the viability of the fetal placental unit. For example, when placentitis is present progestagens are elevated (10 to 40 ng/ml vs. normal of 4 to 10 ng/ml) and total estrogens are lower than normal for that stage of gestation. The level of total estrogens is perhaps the most important for assessing placental damage. The lower concentration the greater the placental damage. Exogenous estrogen therapy is often used to manage these cases along with appropriate antibiotic therapy with a positive outcome. Progesterone assays are also very useful to identify mare that have low-grade endometritis.



## Improving postcryopreservation survival capacity: an embryo approach

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**Keywords:** cryotolerance, *in vitro* produced embryo, lipid.

The major obstacle for a greater dissemination of *in vitro* produced (IVP) bovine embryos is their high sensitivity to the cryopreservation. The modest results of IVP embryo cryopreservation impair the commercialization of embryos between countries and limit its application at the field conditions, as is done with the semen in the artificial insemination. The involvement of embryo lipids on this aspect is well documented. However, it has been recognized that is not only the amount of lipids that affects cryotolerance, the embryo survival capacity after cryopreservation is a multifactorial event. The most common action to deal with these lower results of IVP embryo cryopreservation is to vary the cryopreservation techniques and procedures. Despite this approach usually results in improvements, they often are limited, which suggests to modify the embryos themselves to make them more cryopreservable. Generally, the use of a serum-free media to reduce the lipid content and increase embryo survival after cryopreservation is the first recommended strategy. It has already been described that is possible to produce embryos in defined or semi-defined serum-free media without affecting blastocyst yield. Another approach would be the use of chemicals to modulate lipid metabolism. The addition of phenazine ethosulfate in the post-compaction period reduced the lipid accumulation and increased the postcryopreservation survival. Forskolin, a stimulator of lipase activity, has been used in the culture media to reduce lipid content and increase cryotolerance of bovine and porcine IVP embryos. More recently, L-carnitine has been describe as a chemical candidate for a non-invasive improvement of cryotolerance and developmental competence of IVP embryos because of its unique dual effects that enriches cellular lipid metabolism and provides antioxidative protection. Other strategy described in the literature is the modulation of embryo cell membrane fluidity by cholesterol or unsaturated fatty acids incorporation through its supplementation in the culture media, and oocyte/embryo donor nutritional management. While the addition of cholesterol-loaded methyl- $\beta$ -cyclodextrin to the cryopreservation media had no effect on cryopreserved IVP bovine blastocysts, it seems to have a positive effect on vitrified oocytes. In addition, the unsaturated fatty acid supplementation in the culture media improved the cryotolerance and reduced lipid content of IVP embryos. Likewise, the oocyte/embryo donor nutritional management with a diet enriched in polyunsaturated fatty acids increased the cryosurvival of ewe oocytes and porcine embryos. Therefore, the use of a serum-free media, the addition of chemicals to change lipid metabolism, and the modulation of membrane lipid composition are described as alternatives to modify the embryos themselves and to make them more cryopreservable.



A001 Male Reproductive Physiology and Semen Technology

**Testicular mass and height of the seminiferous epithelium during the postnatal development of guinea pig (*Cavia porcellus*, Linnaeus, 1758)**

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**Keywords:** morphometry, non pubescent, pubescent.

Quantitative parameters relating to the seminiferous tubule have positive relationships with the spermatogenic activity. They are also crucial for studies involving reproductive parameters, once they are constituted into an accurate instrument for evaluating the spermatogenic capacity, both in normal as in pathological or experimental conditions (Castro et al., 1997). Although guinea pigs are excellent experimental models, studies describing quantitative parameters of their seminiferous tubule are nonexistent in this specie. Thus, the objective of this study was to evaluate the seminiferous epithelium height (EH) of guinea pigs during the postnatal development, as contribution to studies focused on their spermatogenic capacity and use as an experimental model. This study was approved by the CEEHA<sup>1</sup> from UNIVASF (protocol nr.22041019). Guinea pigs in the non pubescent (IP), early prepubescent (EP), late prepubescent (LP), pubescent (PU), post pubescent 1 (PP1) and post pubescent 2 (PP2) stages were used (N= 5 animals/group; Gradela et al., 2012, Proceedings...AAAA, 8<sup>th</sup>). Testicular mass (TM; g) was evaluated and the right testicle (N = 3/group) collected, fixed in buffered formalin at 10% for 18 hours and immersed in alcohol 70% for routine histological processing by HE stain. The EH was measured in 10 cross-sections of seminiferous tubules for every animal with full spermatogenic activity (PU, PP1 and PP2), and 20 cross-sections for those that did not have all cells of the spermatogenic lineage (IP, EP and LP). Measurements were performed with 40x micrometric lenses and 40x magnifications, from the basal membrane to the luminal edge. Images were analyzed with the Motic Image Plus 2.0 ML software. Averages were evaluated by ANOVA followed by Tukey test, and simple correlation coefficients (r) were analyzed among the variables (Assistat 7.6 beta). CM increased from 0.03±0.00 g to 1.07±0.17 g, with evident increase (P<0.05) from IP to PP1 stage (0.03±0,00 in IP; 0.16±0,08 in EP; 0.29±0.07 in LP; 0.54±0,14 in PU and 0.87±0,17 in PP1) and discrete increase (P>0.05) in PP2 (1.07±0.17). EH varied from 20.94±4.09 µm in the IP stage to 80.9±11.84 µm in PP1, differing significantly in LP (34.12±11.07 µm); PU (63.12±12.98 µm) and PP1 (80.90±11.84 µm). Average EH of PU, PP1 and PP2 guinea pigs is within the range observed in domestic species (60 to 100 µm; França; Russell. Male reproduction: a multidisciplinary overview, 1998). It may be concluded that the increased EH followed the testicular mass increase, reflecting the different stages of testicular development and coinciding with the full establishment of spermatogenesis that occur at puberty. This knowledge supports the use of guinea pigs as experimental models, since it helps to understand its spermatogenic process.

<sup>1</sup>Comitê de Ética em Estudos Humanos e Animais (Ethics Committee for Human and Animal Studies).



A002 Male Reproductive Physiology and Semen Technology

**Gonadosomatic index and diameter of the seminiferous tubules during the postnatal testicular development of guinea pigs (*Cavia porcellus*, Linnaeus, 1758)**

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**Keywords:** guinea pig, testicular development, testis morphometry.

Testicular morphometric analyses are important for the description of the spermatogenic process of each species, and all quantitative parameters relating to the seminiferous tubule have positive relationship with the spermatogenic activity (França & Russell. In: Regadera & Martinez-Garcia Male reproduction: a multidisciplinary overview. Madrid: Churchill Livingstone, 1998. 198-219). The use of guinea pigs as an experimental model has great importance in scientific research but analysis of its testis morphometry is scarce. The objective of this study was to evaluate the gonadosomatic index ( $GSI (\%) = TM/CM (g) \times 100$ ) and the diameter of the seminiferous tubules (TD) of guinea pigs in the inpubescent (IP), early prepubescent (EP), late prepubescent (LP), pubescent (PU) and postpubescent 1 (PP1) stages (N= 5 animals/group) (Gradela et al., Proceedings... AAAA, 8<sup>th</sup>, 2012). The right testicle (N= 3/group) was collected, fixed in buffered formalin at 10% for 18 hours and immersed in alcohol 70% for histological processing and staining by HE. TD was evaluated in 10 cross-sections of seminiferous tubules for every animal with full spermatogenic activity (PU and PP1) and in 20 sections for those that did not have all cells of the spermatogenic lineage (IP, EP and LP). Measurements were performed with 10x micrometric lenses and 10x magnification and the images were analyzed with the Motic Image Plus 2.0 ML software. Averages were analyzed by ANOVA followed by Tukey test, and simple correlation coefficients (r) were determined among variables (Assistat 7.6 beta). This study was approved by the CEEHA<sup>1</sup> from UNIVASF (protocol nr.22041019). The CM (112.25±1.52 in IP; 225.49±18.53 in EP; 285.04±17.54 in LP; 388.17±29.77 in PU and 471.27±21.32 in PP1) and TM (0.03±0.00; 0.16±0.08; 0.29±0.07; 0.54±0.14 and 0.87±0.17, respectively) increased with age, reflecting the different stages of the testicular development of guinea pigs. Significant correlations (P<0.01) were observed between age and CM (r= 0.95), age and TM (r= 0.94) and CM and TM (r= 0.94). GSI was 0.03±0.00% in IP; 0.06±0.04% in EP; 0.10±0.02% in LP; 0.14±0.04% in PU and 0.17±0.04% in PP1. Average TD varied from 70.97±8.44 to 196.60±29.92, differing (P<0.05) among LP (124.51±20.96); PU (177.63±30.45) and PP1 (196.60±29.92) and had a positive correlation with age. In conclusion, in guinea pigs, the GSI had significant and positive correlations with the TM at puberty and postpuberty<sup>1</sup>, reflecting the reproductive activity and testis growth, coincident with the full establishment of spermatogenesis at puberty. This knowledge helps to understand the spermatogenic process of guinea pigs and assist in its use as an experimental model.

<sup>1</sup>Comitê de Ética em Estudos Humanos e Animais (Ethics Committee for Human and Animal Studies).



A003 Male Reproductive Physiology and Semen Technology

**The extender osmolarity changes the percentage of hyperactive cells in refrigerated boar semen**

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**Keywords:** Osmolarity; hyperactivation; spermatozoa

Hyperactivation must have its beginning in appropriated time and place for the spermatozoa to be able to perform fertilization. Also, the hyperactive sperm movement characteristics are represented by the increase in ALH (amplitude of lateral head displacement) and VCL (curvilinear velocity). The changes in osmolarity affect in greater proportion the motility, when compared to other parameters related to quality and sperm viability (Yest et al, 2010, Anim Reprod Sci, 119, 265-74). Thus, this study verified that different osmolarity conditions modify the percentage of hyperactive spermatozoa. Were made three semen collections of three boars (n=9). Samples were extended in media with various osmolarities, namely 360 and 404 mOsm (Botupharma<sup>®</sup>, Botucatu-SP, Brasil), that differ by changing the dilution, 1.1 L and 1 L of ultrapure water, respectively. Raw semen was mixed within extender to obtain a concentration of  $30 \times 10^6$  spz/ml. Analysis were made at 90 minutes, 24 and 48 hours after dilution. Aliquots of semen were evaluated under cover slide, in computer assisted sperm analysis (SCA-Microptic<sup>®</sup>, Microptic SL, Barcelona, Spain) under an epifluorescence microscope (Nikon, model Eclipse Ni-U). Adjustments included edit/sort toll for the ALH > 3.5  $\mu\text{m}$  and VCL > 97  $\mu\text{m/s}$  values (Schmidt, 2004, Reproduction, 128, 171-79), to evaluate the percentage of hyperactive swine spermatozoa. The experimental design was in generalized blocks, added to repeated measurements in time. Data were analyzed by SAS program (SAS Institute Inc., 2010), subjected to analysis of variance and interactions by Greenhouse-Geisser test at 5%. There was no interaction between time and treatment ( $P > 0,05$ ), for all variables. However, there was a significance trend ( $P=0,0582$ ) to the treatment effect for the sperm hyperactivation, with values as  $16,66 \pm 2,44$  and  $24,79 \pm 3,46$  to the 360 and 404 mOsm treatments, respectively. This results lead us to believe that extenders with greater osmolarity, as represented by the 404 mOsm extender, tends to show induce a higher percentage of hyperactive cells. Such results can be verified in further studies, with a greater number of repetitions.

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A004 Male Reproductive Physiology and Semen Technology

**Cooling and cryopreservation effect on viability of ejaculated and epididymal spermatozoa of Gir bulls**

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**Keywords:** bovine, epididymis, spermatozoa.

The use of epididymal spermatozoa in various assisted reproductive technologies is an important tool to multiply genetic material from animals that die suddenly and/or have acquired reproductive failure. However, to establish more appropriate procedures to use spermatozoa from epididymis, it is necessary to know about their behavior and viability after stress situations such as cooling and cryopreservation. To evaluate the characteristics and viability of epididymal and ejaculated spermatozoa, seven bulls Gir were used. Ejaculated semen (EJ) was collected by electrostimulation. After collecting the ejaculate, the animals were castrated and the testicles were kept for two hours at 5°C, simulating the transport to the laboratory. Then, spermatozoa from the cauda epididymis were collected by extravasation (PE) method. In both groups, sperm samples were diluted in tris-yolk, packaged and refrigerated for four hours at 5°C and then cryopreserved. After cooling and thawing, samples were taken for evaluation of total and progressive motility in a computerized system (CASA), plasma membrane integrity, assessed with propidium iodide (PI) and 6-carboxyfluorescein diacetate (C-FDA) and acrosomal integrity by the technique of Peanut agglutinin (PNA conjugated to FITC). Data were analyzed by ANOVA and Tukey test ( $P < 0.05$ ). After cooling, epididymal sperm had higher total motility, progressive motility and acrosome integrity than the ejaculate, being  $58.9 \pm 21.2\%$  and  $79.5 \pm 5.9\%$ ,  $29.6 \pm 13.9\%$  and  $46.1 \pm 7.2\%$ ,  $48.9 \pm 20.6\%$  and  $69.4 \pm 7.3\%$ , respectively. However, the percentage of cells with intact plasma membrane was similar between the two groups (EJ =  $56.4 \pm 16.6\%$  and PE =  $69.7 \pm 8.8\%$ ). After thawing, no difference was found in any of the characteristics studied: total motility, progressive motility, acrosome integrity and plasma membrane integrity, with  $48.4 \pm 9.8\%$  and  $54.2 \pm 10.7\%$ ,  $26.3 \pm 8.8\%$  and  $35.4 \pm 11.3\%$ ,  $41.3 \pm 12.9\%$  and  $46.8 \pm 7.6\%$  and  $41.6 \pm 11.9\%$  and  $50.8 \pm 9.0\%$  for the EJ and PE, respectively. The results indicate that epididymal spermatozoa are more resistant to cooling than EJ, but behave similarly when submitted to cryopreservation. However, more studies are needed to evaluate other sperm characteristics, such as longevity, capacitation, response to sperm selection for IVF and binding capacity to the zona pellucida, among others.



A005 Male Reproductive Physiology and Semen Technology

**The influence of brucellosis on the morphological characteristics of canine epididymal sperm**

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**Keywords:** brucellosis, dog, epididymal spermatozoa.

Canine Brucellosis is a zoonosis of difficult epidemiological control, can partially compromise fertility and has limited diagnostic definition. The reproductive analysis of a mongrel dog, 2 years old, identified *Brucella canis* infection, through the test named Canine Brucella Ab Rapid Antigen Test (Bioeasy, Minas Gerais, Brazil), which is highly specific for this agent. The physical examination revealed no remarkable change and the dog was submitted to orchiectomy. At macroscopic exam, the testis and epididymides were normal. Due to the frequent location of the bacteria in the epididymis, sperm were harvested from the caput (CP), body (BO) and tail (TA) of the epididymides and evaluated for sperm concentration, motility and morphology (eosin/nigrosin stain), mitochondrial activity (oxidation of 3,3'-diaminobenzidine, DAB), acrosomal membrane integrity (Fast Green-Rose Bengal stain) and flow cytometry with the probes JC1 (mitochondrial potential), FITC (acrosomal membrane integrity) and PI (plasma membrane integrity). The results were analyzed based on the sperm evaluation of 20 serologically negative dogs. The concentration and sperm motility of the tail were lower ( $325 \times 10^6$  sperm/mL and 50%, respectively) than the serologically negative dogs ( $866 \pm 129 \times 10^6$  sperm/mL and  $72 \pm 2\%$ ). There was a lower mitochondrial activity and potential due to the infectious process in the three epididymal segments (DABIII CP: 20%, BO: 20% and DABIV TA: 20%, control: DABIII CP:  $13 \pm 1\%$ , BO:  $9 \pm 1\%$  and DABIV TA:  $10 \pm 5\%$ ; JC1 CP: 20%, BO: 11% and TA 10%, control: CP:  $13 \pm 1\%$  BO:  $40 \pm 3\%$  and TA:  $65 \pm 4\%$ ). A higher percentage of acrosome membrane lesions were found in the body and tail samples (BO: 40%, TA: 11%, control BO:  $22 \pm 4\%$  and TA:  $7 \pm 1\%$ ), teratospermia (major defects: 29%, control:  $19 \pm 5\%$ ), and high frequency of sperm distal droplets on the epididymal tail (21%; Control:  $6 \pm 1\%$ ). Despite the absence of clinical signs and macroscopic alterations of the testis and epididymides, sperm evaluations revealed important data. Alterations of the sperm derived from the epididymal tail suggest that the severity is associated with the increased temporal exposure to the epididymal environment, by means of bacterial toxins or through the local immune response. Due to the inflammatory changes promoted by the bacteria, there was an increased production of free radicals and, consequently, a reduction of sperm mitochondrial activity. The decrease in sperm motility can be attributed to the lower energy production by mitochondria. Lipid peroxidation justifies the changes found in acrosome membrane. The high percentage of distal droplets in epididymal tail spermatozoa may reflect a delayed maturation, probably as a consequence of Brucellosis. *Brucella canis*, though insidious, promotes important sperm changes in dogs, which can serve as a definitive diagnosis, along with the results of canine Brucellosis serology.



A006 Male Reproductive Physiology and Semen Technology

### **Sperm profile during epididymal maturation in dogs**

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**Keywords:** epididymis, dogs, sperm maturation.

Sperm maturation through the epididymis is a process that leads to morphologically and functionally changes, enabling fertilization. Therefore, major elucidations regarding the physiology of sperm maturation are necessary for further improvement of biotechnologies uses with epididymal sperm. Thus, the aim of this study was to determine the spermiatic profile of canine epididymal samples. We used 21 dogs submitted to bilateral orchiectomy. Until processing, the epididymides were stored at 5°C for up to 24 hours. Sperm were harvested through small incisions (<1mm) in the caput (CAP), corpus (COR) and tail (TAIL) of the epididymides and then deposited in 300µl of PBS. Samples were evaluated for motility, velocity and Computer Assisted Sperm Analysis (CASA). To evaluate the plasma membrane permeability, acrosomal integrity and mitochondrial activity, we performed the eosin/nigrosin, fast green/rose bengal and the oxidation of 3,3'-diaminobenzidine (DAB) stains, respectively. In order to determine plasma membrane integrity, acrosomal and mitochondrial potential, we used flow cytometry with specific probes (FITC, PI and JC1, respectively). Data were compared by ANOVA and Tukey test ( $p \leq 0.05$ ). Regarding sperm motility and velocity, statistical difference was noticed between the samples of TAIL (mot: 69.7±4%; velocity: 2.6±0.1), compared to COR (mot: 27.7±3%; velocity: 2±0.1), both higher than CAP (mot: 0±0%; velocity: 0±0). The same data pattern was observed for the CASA variables: motile, progressive, rapid, VAP, VSL, straight trajectory and linear. In regards to the percentage of intact plasma membranes, TAIL samples (92.6±1.1%) were higher than COR (74.5±3.2%), which in turn was higher than the CAP (40.1±4.7%). The highest mitochondrial activity (DAB I) was observed for TAIL (75.9±3.4%), with values greater than COR (46.8 ± 2.5%), the latest superior than CAP (26±2.4%). Acrosomal damage was higher in CAP samples (40.2±3.7%), compared to COR (14.4±1.1%) and TAIL (6.5±1.8%). For flow cytometry, we observed lower plasma membrane acrosomal integrity in the COR (20.6±2.6%) and CAP (24.6±2.4%) groups, compared to the TAIL group (38.5±3.4%). Moreover, we found increased mitochondrial potential in TAIL samples (65±4.2%) compared to the COR (38.3±3.6%) and CAP (43.6±4.1%). In conclusion, sperm from the epididymis tail have a higher sperm profile than corpus and caput groups, especially regarding sperm membrane integrity, motility and, consequently, the fertilizing capacity. Our results confirm the occurrence of morphological and functional changes during the transit through the epididymides, enabling the use of epididymal sperm in several reproductive biotechnologies.



A007 Male Reproductive Physiology and Semen Technology

**Scrotal thermography in rams after testicular degeneration and low level laser therapy treatment: preliminary results**

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**Keywords:** insulation, laser therapy, thermography.

The body surface emits radiation in the infrared range of the electromagnetic spectrum. The thermographic camera is able to capture the radiation and produce images with different colors that depend of the quantity of radiation emitted. Thus, the thermography is a helpful tool to measure the scrotal surface medium temperature (SSMT). The low level laser therapy (LLLT) induces cellular proliferation and it is possible that LLLT contributes to the restoration of the seminiferous epithelium after testicular degeneration. The biostimulation was observed in the seminiferous epithelium of rats (Taha and Valojerdi, 2004, *Lasers Surg. Med.*, 34, 4, 352-359); however, the temperature of the treated area may increase until 0.5°C. Thus, the aim of this study was to evaluate the SSMT of rams submitted to scrotal insulation for the induction of testicular degeneration. For this, six rams were divided in three experimental groups: 1) Control, without LLLT treatment (n=2); 2) LLLT treatment of cumulative dose of 28J/cm<sup>2</sup> (n=2); 3) LLLT treatment of cumulative dose of 56J/cm<sup>2</sup> (n=2). It was used the output power of 30 mW and the treatment period was of 15 days, every 48h, in groups 2 and 3. Scrotal insulation was done in all rams for 72h before the experimental period. The thermal images were captured in at: T-8, T-5, T-1, T0, T3, T4, T6, T10, T17, T24 and T31 days, as related to insulation, being T0 the time that corresponded to placing the insulation bags. A T640 thermographic camera was used (FLIR Systems, Boston, USA) and thermal images were analyzed by the FLIR Quick Report<sup>®</sup> Software. The environmental temperature was measured at the moment of image acquisition. SSMT values were corrected by the environmental temperature (Basile et al., 2010, *ARS Veterinária*, 26, 77-81). The results were analyzed employing the Statistical Analysis System (SAS Institute Inc., 1995). The SSMT data were submitted to ANOVA and the factor repeated measures in time was added. Tukey's test was used to compare the averages when there was time X treatment interaction or time and treatment effect. There is no time X treatment interaction (P=0.48) neither treatment effect (P=0.25). However, it was observed a time effect (P=0.001). The SSMT was significantly (P>0.05) higher in T3 (36.25±0.49°C) than T-8 (33.72±0.27°C), T-5 (33.70±0.30°C), T-1 (33.87±0.41°C), T0 (33.49±0.24°C), T6 (32.95±0.26°C), T10 (33.83±0.24°C), T17 (33.76±0.34°C), T24 (33.17±0.36°C) and T31 (32.78±0.53°C). T3 was the time immediately after removal the insulation bags. On the other hand, T3 was not different (P>0.05) of T4 (34.75±0.24°C). In conclusion, thermography is applicable to the evaluation of the SSMT in insulated rams and LLLT does not change the SSMT of treated groups. However, the results are preliminary and more studies are needed.

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A008 Male Reproductive Physiology and Semen Technology

**The effect of seminal plasma removal on cryopreservation of semen from Nelore bulls collected by eletroejaculation using different methods of separation (preliminary data)**

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**Keywords:** centrifugation, computerized evaluation of sperm, filtration, seminal plasma.

Seminal plasma, that mixes with sperm in the ejaculate, and serves as a means of transportation to the female genital tract, has been described as beneficial and harmful to the spermatozoa. There are reports of negative influence of seminal plasma on the storage of semen, due harmful components to sperm viability. An alternative to reduce the concentrations of seminal plasma of the ejaculate is semen centrifugation or filtration. However, several studies have reported apparent injury to bovine sperm, damaging fertilization, by the method of centrifugation. The objective of this study was to evaluate the frozen-thawed semen from 13 Nelore bulls collected by electroejaculation with the removal of seminal plasma through centrifugation and filtration techniques and their consequences on sperm kinetics. The work is being conducted at the Institute of Animal Science Sertãozinho / SP (partial data). Semen was collected using electroejaculation and were performed the physical and morphological analyzes routine before freezing (volume, motility, vigor, concentration and morphology). After collection, ejaculated semen was divided into three treatments before freezing: standard freezing T1, T2 and T3 were used to separate the seminal plasma. T2 semen was centrifuged (10 minutes at 600 xg) and T3 semen was filtered using the Filter Sperm<sup>®</sup>. After treatments, semen was diluted with BotuBov<sup>®</sup> extender to a final concentration of 100x10<sup>6</sup> sperm/ml and frozen using a freezing machine TK 4000<sup>®</sup>. Semen was thawed at 37 °C for 30 seconds and analyzed by Hamilton Thorne Research - IVOS 12, placing a drop in the Makler chamber heated to 38 °C for the analysis. Sperm variables considered were: total sperm motility (TM), motility (PM), average path velocity (VAP), progressive velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), tail beat frequency (BCF), straightness (STR) and linearity (LIN). The statistical analyzes were performed in SAS PROC GLM, using 5% significance. Mean values for MT (T1, 39.5 ± 4.0, T2, 39.3 ± 4.0, T3, 42.6 ± 4.0), MP (T1, 29.8 ± 2.8, T2, 28.0 ± 2.8, T3, 33.0 ± 2.8), STR (T1, 83.9 ± 1.1, T2, 81.3 ± 1.1, T3, 83.3 ± 1, 1), ALH (T1, 5.3 ± 0.2, T2, 6.1 ± 0.2, T3, 5.9 ± 0.2) and LIN (T1, 55.3 ± 1.5, T2; 52.2 ± 1.5, T3, 53.6 ± 1.5) did not differ between treatments (P>0.05). However, for VAP and VCL, T2 (88.2 ± 2.3 146.3 ± 5.4) and T3 (88.9 ± 2.3 145.0 ± 5.4) differed from T1 (81 10 ± 2.3 130.6 ± 5.4), respectively (P <0.01). For VSL, T3 (74.1 ± 1.5) differed from T1 (67.8 ± 1.5; P <0.01). Based on these preliminary results, it can be concluded that frozen semen after removal of seminal plasma by centrifugation and filtration were better for VAP, VSL and VCL compared to standard freezing.



A009 Male Reproductive Physiology and Semen Technology

**Effect of catalase on freezing of sperm cell obtained from the cauda epididymis of Nelore bulls**

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**Keywords:** antioxidant, cryopreservation, germosplasm.

Previous dilution in semen cryopreservation processes promotes reduction in antioxidants concentration, which may cause imbalance between oxidants and antioxidants concentrations, and cell stress (Bilodeau et al., 2000, Mol Reprod Dev, 55, 282-8). Catalase (CAT) is an enzymatic antioxidant that catalyses H<sub>2</sub>O<sub>2</sub> molecules into water and oxygen (Norderberg, 2001, Free Rad Biol Med, 31, 1287-312). This study aimed to evaluate sperm integrity obtained from cauda epididymis of Nelore bulls subjected to freezing in diluents supplemented with CAT. Epididymides were obtained from slaughterhouse, minutes after animals' death. Sperm cells were recovered by flotation technique (Almeida et al., 2012, Anim Reprod, 9, 959), diluted in Tris-yolk egg. Each *pool* of sperm was formed from five epididymis, with a total of 11 *pools*. Samples were divided into experimental groups (control, CAT 50 and 100 U/mL), with 80x10<sup>6</sup> sperm/mL as the final concentration, packed in straws (0.25 mL) and frozen (TK3000®, TK Tecnologia em congelação LTDA, Uberaba, Brasil). After thawing (37°C/30s), samples were evaluated for motility (CASA/SCA™ software v.5.1; Microoptics, S.L., Barcelona, Spain), plasma membrane integrity (PMi), acrosome integrity (ACi) and mitochondrial membrane potential (MMP). We performed analysis of variance (F test) and test of Student-Newman-Keuls (P < 0.05). All experimental groups demonstrated post-thaw motility above 60.00%. Integrity assessments revealed no significant difference (P > 0.05) between control, CAT 50 and CAT 100 groups, respectively, PMi (49.55±4.46; 48.27±5.53; 50.82±4.89), ACi (32.82±28.34; 51.50±35.82; 53.18±32.66) and MMP (62.86±6.47; 63.73±8.71; 65.05±10.68). Based on results, we conclude that CAT addition at concentrations of 50 and 100 U/mL do not affect the integrity of epididymal spermatozoa.



#### A010 Male Reproductive Physiology and Semen Technology

### Evaluation of canine semen cooled after 120 hours with different means

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**Keywords:** botu-semen, dogs, extenders.

The biotech semen in dogs have had great progress in recent years, due to the demand to improve fertility rates in artificial insemination in this species. The cooled semen has proved the best technique for insemination later, being a practical and low cost. After cooling semen adding extenders becomes important factor for enhancing the viability thereof, for extended periods of time. The aim of this study was to evaluate the quality of semen cooled for 120 hours in three different extenders, one milk-based (Botu-semen<sup>®</sup> -Botucatu/SP, Brazil) and two based on egg yolk and Tris, Tris egg yolk without glycerol (TRIS) buffer and Tris egg yolk with 6% glycerol (TRIS + GL). Were cooled ejaculates of four dogs that after centrifugation for 10min (600G), were fractionated and diluted in the three extenders (Botu-semen<sup>®</sup>, TRIS, TRIS + GL) and assessed every 12 hours to complete the 120 hours. To evaluate sperm parameters were observed: total motility (TM) and progressive (MP), sperm vigor (VIG), morphology and functionality of the membrane hypo osmotic swelling test (HOST). After 120 hours, the evaluations were done and the results demonstrated that TRIS got better indexes in all parameters, in which the MT obtained was 21.3%, 20.0 and 37.5%, for the media Botu-semen<sup>®</sup>, TRIS and TRIS + GL, respectively. The MP was 11.3%, 10% and 27.5% means for Botu-semen<sup>®</sup>, TRIS and TRIS + GL, respectively. The results of VIG were higher for TRIS (2.5) compared to other means Botu-semen<sup>®</sup> (1.3) and TRIS + GL (0.9). The same happened to HOST, in which the group obtained TRIS index with sperm membrane integrity of 39.0%, higher than the results observed means for Botu-semen<sup>®</sup> and TRIS + GL, with 20% and 37.5% respectively. After this study it was concluded that the TRIS provided better maintenance rates of sperm parameters after 120 hours, which proves to be the best alternative among the means evaluated for cooling canine semen.



#### A011 Male Reproductive Physiology and Semen Technology

### The osmolarity of the extender affects the viability of chilled swine spermatozoa?

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**Keywords:** membrane integrity, osmolarity, spermatozoa.

The present study evaluated the effects of two different conditions of osmolality on plasma membrane and acrosomal integrity, and mitochondrial membrane potential. Therefore, three semen collections from three boars were carried out (n = 9). The samples were diluted in media with different osmotic potentials, namely, 360 (A) and 404mOSM (B) (Botupharma<sup>®</sup>, Botucatu-SP, Brazil). The osmotic potentials differed by changing the amount of ultrapure water used for dilution, 1.1 L (360 mOsm) and 1 l (404 mOsm). Fresh semen was mixed with extenders to obtain a concentration of 30 x 10<sup>6</sup> spz/mL. Analyses were performed at 90 minutes, 24 and 48 hours after dilution. They were analyzed for the sperm cells that presented simultaneously plasma membrane integrity, acrosomal and mitochondrial membrane potential (PIAIC) (Celeghini et al., 2007, *Reprod Domest Anim*, 42, 479-88). For this, aliquots of 150µL of semen were stained with Hoescht 33342 (5 mg / ml in DMSO) iodide, propidium (0.5 mg / ml in DPBS), JC-1 (153 mM in DMSO) and 50 l of FITC -PSA (100 g / ml in DPBS). The samples were incubated for 8 minutes at 37°C and then evaluated under epifluorescence microscopy (Nikon Eclipse Model NI-U) in a triple filter (D / F / R, C58420) at 1000X magnification. The experimental design was in generalized randomized blocks added the factor of repeated measures. Data were analyzed using SAS (SAS Institute Inc., 2010), subjected to analysis of variance and interactions by the Greenhouse-Geisser at 5%. Data are presented as mean ± standard error. There was no time x treatment interaction (P > 0.05) for the PIAIC variable. Thus, we assessed the effect of osmolality factor. Apparently, increased osmolality improves the integrity of the plasma and acrosomal membranes as well as mitochondrial membrane potential (p = 0.0571). Extender A showed 54.09 ± 4.14% of cells PIAIC, while extender B had 64.14 ± 3.14%. From these results, it can be concluded that the higher the osmolality, the greater the number of cells with plasma membrane integrity, acrosomal and mitochondrial membrane potential.

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A012 Male Reproductive Physiology and Semen Technology

**Epididymis morphology and morphometry of the *Trachemys scripta elegans* (Wied, 1839) raised in Brazil**

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**Keywords:** Epididymis; turtle; testudines

*Trachemys scripta elegans* (WIED, 1839) (*T. scripta elegans*) is an underwater exotic turtle invasive of the Cerrado<sup>1</sup>, being constantly marketed in Brazil. The function of epididymides is the maturation and storage of spermatozoa, providing a favorable environment to physiological and morphological changes. However, information on this species is scarce, which may assist in population control measures or comparative studies. The objective of this study was to describe the epididymides morphology and morphometry of *T. scripta elegans* raised in Brazil and correlate the epididymal morphometry with body biometric data. Eleven males of the species, from the Parque Ecológico do Tietê<sup>2</sup> (IBAMA<sup>3</sup> Record Nr. 2491988), had the reproductive system and the body biometrics (mass (MC), volume (VC), carapace length (CARL), carapace width (CARW), plastron length (PLAL), plastron width (PLAW) and height (HEI)) assessed. The epididymis were dissected for macroscopic and morphometric evaluation (mass (ME), volume (VE); length (EL), width (EW) and thickness (ET)). The Kruskal-Wallis test followed by t test was employed for comparison of means ( $\pm$  sd) and the simple correlation coefficient (R) was determined among the variables (Assistat 7.6 beta). This study was approved by the CEDEP<sup>4</sup> from UNIVASF<sup>5</sup> (Protocol nr. 0001/160412). Genitals were a pair of testicles and epididymis, vas deferens, mesorchium present throughout the testicular wall and a penis. Epididymis were convolute structures and were located in the right and left antimeres connected to the medial surface of each testicle and joined to vas deferens, which penetrated into the cloaca, near the opening base of the urinary bladder. MC was 587.45 $\pm$ 198.37 g, VC 556.54 $\pm$ 289.75 ml, CARL 15.50 $\pm$ 2.35 cm, CARW 12.2 $\pm$  1.39 cm, PLAL 13.72 $\pm$ 1.93 cm, PLAW 9.21 $\pm$ 1.48 cm and HEI 5.57 $\pm$ 1.34 cm. ME, VE and ET differed (P<0.01) between right and left antimeres (0.28 $\pm$ 0.25 vs 0.29 $\pm$ 0.25 g, 0.24 $\pm$ 0.16 vs 0.26 $\pm$ 0.16 ml and 0.24 $\pm$ 0.23 vs 0.25 $\pm$ 0.20 cm, respectively), while EL and EW did not differ (P>0.05) (1.81 $\pm$ 0.42 vs 1.74 $\pm$ 0.43 cm and 0.52 $\pm$ 0.12 vs 0.55 $\pm$ 0.27 cm, respectively). It is concluded that in this species, body parameters have significant correlation among themselves except for CARL and HEI. Also, ME correlates with the other morphometric parameters in both antimeres, while VE correlates to EW and ET in the right antimere and only with EW in the left antimere. These data provide information on *T. scripta elegans* epididymis and may be helpful in population control and comparative studies.

**References**

<sup>1</sup>Vast tropical savanna Eco region in Brazil; <sup>2</sup>Tietê Ecological Park; <sup>3</sup>Instituto Brasileiro de Meio Ambiente e Recursos Naturais Renováveis (Brazilian Institute for Environment and Renewable Natural Resources); <sup>4</sup>Comitê de Ética e Deontologia (Ethics and Deontology Committee); <sup>5</sup>Universidade Federal do Vale do São Francisco (Federal University of the São Francisco Valley).



A013 Male Reproductive Physiology and Semen Technology

**Flow-cytometer sex sorting affects sperm characteristics related to viability and capacity to bind oviduct cells**

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**Keywords:** bovine, fertility, fertilization.

This study assessed the effect of sex process on sperm survival and capacity to bind to oviduct cells, which are essential for IVF. Each ejaculate of Nelore bulls (n=4) was collected and separated into three fractions: non-sexed (NS), sexed for X-sperm (X), and sexed for Y-sperm (Y). A fourth group was formed by pooling X and Y samples (XY). Semen from each group was assessed for sperm viability after thawing (0-), after washing (0) and 2, 4, 8 and 12 h after incubation in synthetic oviduct fluid. In each moment, the samples were analyzed for sperm motility by computer-assisted semen analysis (CASA), plasma membrane stability (PM, merocyanine 540), PM integrity (Syber green), acrosomal integrity (PNA) and mitochondrial membrane potential (Mitotracker green) in flow cytometer. For sperm binding test, sperm from each group/bull was incubated for 30 min and 24 h with oviduct explants. Data were analyzed using generalized linear models (SAS<sup>(R)</sup>; P<0.05). The percentages of sperm motility (58.1±4.3 and 35.2±4.4), progressive motility (46.1±6.1 and 25.7±4.8), PM integrity (91.0±3.9 and 79.5±6.0), mitochondrial membrane potential (79.2±9.3 and 69.0±10.6), PM stability (77.4±4.6 and 19.4±4.2), and live sperm with intact acrosome (57.2±8.5 and 31.3±7.9) were higher in non sexed than in sexed sperm. Moreover, percentages of sperm with destabilized PM and live sperm with reacted acrosome were higher in sexed sperm (65.7±5.5 and 35.6±8.9) than in non sexed (2.5±0.9 and 15.7±3.8). Those differences were kept up to 8 h of incubation. However, after 12 h sperm quality was similar between groups. By comparing the X and Y groups it was observed that after wash the X sperm (56.6±13.9) had higher percentage of sperm with mitochondrial potential than Y sperm (41.7±13.8), and higher percentage of live sperm with reacted acrosome at 0, 2 and 4 h of incubation. The sexing process did not affect sperm binding to the oviduct cells after 30 min. However, after 24 h, the XY group (6.7±2.0) had less sperm bound in oviduct explants than NS group (23.6±7.2). After 30 min, the Y group (84.9±17.0), showed higher number of sperm bound than the X group (72.1±20.3), but there was no difference between them at 24 h. In conclusion, sexing process affected structural characteristics of the sperm cell, inducing a precapacitation status and acrosome reaction as well as its capacity to remain bound to the oviduct explants. Regarding to X and Y sperm, it was identified that X sperm was more sensitive to the sexing process than Y sperm. Those pieces of information can be used to develop new alternatives to improve the results when sexed sperm is used for AI. The alternatives can be changes in protocols for AI, such as different moments for insemination, or in cryopreservation processes, such as adding protein extract of seminal plasma after sexing to increase the capacity of the sperm to remain bound to oviduct cells.

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A014 Male Reproductive Physiology and Semen Technology

**Standardization of sperm binding assay in cryopreserved bull semen evaluated by computer assisted sperm analysis and conventional microscopy**

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**Keywords:** bull semen, functional tests, sperm interaction.

The interaction between spermatozoa and zona pellucida occurs through binding of sperm acrossomal receptors to zona pellucida ZP3 and ZP2 proteins (Yanagimachi, 1994, *Zygote*, 2, 371-72). The perivitelline membrane of chicken eggs shows homologies with these proteins, allowing the binding of sperm from several species (Barbato et al., 1998, *Biology of Reproduction*, 58, 686-99). However, adequate standardization is required in order to verify the possible application of this technique for semen evaluation in determined species. The present study aimed to validate the sperm binding assay in cryopreserved bull semen by evaluating the results of the test using different proportions of live / dead sperm (0%, 25%, 50%, 75% and 100%). Cryopreserved samples (n=6) were thawed and divided in two aliquots; one kept in water bath at 37°C (live) and the other immersed in liquid nitrogen and thawed 3x (dead). Both samples were then mixed in different proportions which were then submitted to computerized motility analysis (CASA; Hamilton-Thorne Ceros 12.3), membrane integrity (eosin / nigrosin), acrosomal integrity (Fast-green/Rose-bengal) and mitochondrial activity (3'3 Diaminobenzidine) evaluations. Shortly thereafter, membrane squares measuring 0,5 cm<sup>2</sup> were incubated with the live/dead proportions in a concentration of 100.000 spermatozoa per ml, at 37°C for one hour. Sperm binding was assessed by both conventional microscopy and CASA. Results were expressed as the number of sperm bound per mm<sup>2</sup> of membrane. Statistical analysis used SAS System for Windows. With the increase in the proportion of live sperm, there was an increase in the number of spermatozoa bound to the membrane, in evaluations performed using both CASA and microscopy. Linear regression showed a high relationship between the proportion of live sperm and the number of sperm bound to the membrane (CASA, R<sup>2</sup> = 0.92, p <0.0001, Microscope, R<sup>2</sup> = 0.91, p <0.0001). There was a positive correlation between the number of spermatozoa bound to the membrane and acrosomal integrity (% CASA 0.846, p <0.0001; Microscope 0.788, p <0.0001) membrane integrity (%CASA 0.845, p <0,0001; Microscope 0.846, p <0.0001), high mitochondrial activity (%; CASA 0.759, p <0.0001; Microscope 0.768, p <0.0001), motility (%; CASA 0.890, p <0.0001, Microscope 0.806 p <0.0001) and progressive motility (%; CASA 0.861, p <0.0001, Microscope 0.919, p <0.0001). We can conclude that the sperm binding assay may be used in for cryopreserved bull semen as an alternative tool to indirectly assess several sperm functions, with affordable costs and straightforwardness methodology.



#### A015 Male Reproductive Physiology and Semen Technology

### Effect of busulfan on spermatogonial markers expression in different mouse strains

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**Keywords:** gene expression, spermatogonia stem cells, testicular degeneration.

Spermatogenesis is a complex process where many spermatozoa are produced from a small number of spermatogonial stem cells (SSC), which are responsible for the maintenance and constant sperm production throughout the male life (Brinster and Zimmermann, 1994, PNAS, 91, 11298-302). Although the gene expression pattern of spermatogonial stem cells is not completely elucidated, some fundamental genes related to the maintenance and self-renew of these cells and some specific molecular markers are well established (Tsuda et al., 2003, Science, 301, 1239-41; Costoya et al., 2004, Nat Genet, 36, 653-59; Meng et al., 2000, Science, 287, 1489-93). Among the various models used in testicular degeneration studies, one of the most utilized is the administration of busulfan (Zohni et al., 2012, Hum Reprod, 27, 44-53). However, the effect of busulfan treatment on the expression of spermatogonial molecular markers in mice has not been established yet. The aim of this study was evaluate the mRNA expression of spermatogonial markers (*Nanos2*, *Nanos3*, *GDNF* and *PLZF*) after busulfan treatment in two different mice strains. Balb/C and Swiss male mice were used in this study. Animals of each strain were randomly divided in control groups C30 and C90 (30 and 90 days after vehicle administration) and into busulfan treated groups (B30 and B90, 30 and 90 days after busulfan administration). Animals from busulfan treated groups receive a unique intraperitoneal injection (i.p.) of 40 mg kg<sup>-1</sup> of busulfan, while animals from control group receive just vehicle. After 30 and 90 days of the busulfan treatment, the animals were, anesthetized and had the testes surgically removed. Total RNA was extracted from the testes using Trizol and the mRNA expression analyzed by qRT-PCR. The mRNA expression was corrected by amplification of *Gapdh* housekeeping gene. Gene expression were analyzed by ANOVA and means compared by a Student's *t* test. A significant reduction in *Nanos2* mRNA expression was observed at 30 (P<0.01) and 90 days (P<0.05), whereas the expression of *Nanos3* mRNA was only observed at 30 days after busulfan administration (P<0.001) in treated Balb/C mice. The *Gdnf* and *Plzf* mRNA levels increased by more than 2.0-fold, independently of the time lapse between busulfan treatment and control group in Balb/C (P<0.05). On the other hand, the expression of *Nanos2*, *Nanos3* and *Plzf* mRNA revealed no statistical differences between the experimental groups in Swiss mice (P>0.05). However, the *Gdnf* mRNA levels increased after 30 days of busulfan treatment in Swiss animals (P<0.05). This study demonstrated that busulfan changes the expression of spermatogonial molecular markers probably depending on cell type present in the seminiferous tubules and these alterations are dependent on mice strain.



#### A016 Male Reproductive Physiology and Semen Technology

### Osmolarity and motility characteristics of swine sperm: changes in characteristics of sperm movement

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**Keywords:** motility, osmolarity, spermatozoa.

Motility is the most affected parameter by variations in osmolarity (Yest et al., 2010, Anim Reprod Sci, 119, 265-74), and osmotic tolerance is a potential indicator of sperm function (Lechniak et al., 2002, Redrod Domest Anim, 37, 379-80). Thus, this study evaluated the effects of different osmolarities of boar extender in sperm characteristics: total motility (MT,%), progressive motility (MP,%), average path velocity (VAP,  $\mu\text{M} / \text{s}$ ), straight velocity (VSL  $\mu\text{M}/\text{s}$ ), curvilinear velocity (VCL,  $\mu\text{M}/\text{s}$ ), amplitude of lateral head displacement (ALH,  $\mu\text{M}$ ), beat cross frequency (BCF, Hz), straightness (STR,%) and linearity (LIN,%). Were made three semen collections of three boars (n=9). Samples were extended in media with various osmolarities, namely 360 (T1) and 404 (T2) mOsm (Botupharma<sup>®</sup>, Botucatu-SP, Brasil), that differ by changing the dilution, 1.1 L and 1 L of ultrapure water, respectively. Raw semen was mixed within extender to obtain a concentration of  $30 \times 10^6$  spz/mL. Analyses were made at 90 minutes, 24 and 48 hours after dilution. Aliquots of semen were evaluated under cover slide, in computer assisted *sperm* analysis (SCA-Microptic<sup>®</sup>, Microptic SL, Barcelona, Spain). The experimental design was in generalized blocks added to measures repeated in time. The data were analyzed by SAS program (SAS Institute Inc., 2010), subjected to analysis of variance and interactions by Greenhouse-Geisser test at 5%. There was no interaction between time and treatment ( $P>0.05$ ; T1 and T2) for any of the variables (MT, MP, VAP, VSL, VCL, ALH, BCF, STR, LIN). However, a treatment effect ( $P<0.05$ ) for the characteristics VAP ( $33.27 \pm 1.67$ ,  $38.77 \pm 1.97$ ), VCL ( $58.12 \pm 2.8$ ,  $68.81 \pm 3.04$ ), STR ( $58.55 \pm 1.65$ ,  $49.04 \pm 1.41$ ) and LIN ( $33.03 \pm 1.89$ ,  $27.78 \pm 1.18$ ) were found. A significance trend ( $p = 0.0548$ ) for the ALH variable with values of T1:  $2.53 \pm 0.1$  and T2:  $2.83 \pm 0.11$ . Nevertheless variables MT ( $89.92 \pm 2.64$ ,  $91.31 \pm 1.21$ ), MP ( $73.33 \pm 4.07$ ,  $76.35 \pm 3.27$ ), VSL ( $19.61 \pm 1.09$  and  $18.4 \pm 0.78$ ) and BCF ( $8.75 \pm 0.43$ ,  $9.3 \pm 0.25$ ) not differ ( $P> 0.05$ ) between T1 and T2 respectively. Thus, changes in osmolarity did not alter ( $P>0.05$ ) motility characteristics, but extender with 404 mOsm increase VAP, VCL and ALH, while LIN and STR decrease. The 404 mOsm extender interferes in different characteristics (VAP, VCL, ALH, LIN, STR) suggest that it increases the number of hyperactive cells. Hypermotility as well sperm capacitation should occur at a favorable time and local, since that the beginning of these processes by sperm cells reduce their survival in the female reproductive tract, thus leading to a possible decrease in fertility.

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A017 Male Reproductive Physiology and Semen Technology

**New insights on the use of soybean lecithin for cryopreservation of bull semen**

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**Keywords:** antioxidant, bull, sperm cryopreservation.

Nowadays, significant discussion exists about the use of seminal egg yolk-based extender because of bacteriological contamination risks (Gil et al., 2003, *Theriogenology*, 59, 1157–70). However, products that could replace the egg yolk on sperm extenders do not show satisfactory results (Celeghini et al., 2008, *Anim Reprod Sci*, 104, 119-31). With this in mind, the aim of the present experiment was to compare the effects of egg yolk-based extender and soybean lecithin-based extender, supplemented with two types of antioxidants, on maintenance of bovine cryopreserved sperm samples. Seminal samples from 20 Brangus bulls were collected by electroejaculation and samples were divided in 3 extenders groups: LA- soybean lecithin supplemented with ascorbic acid (AA, 4.5mM); LS- soybean lecithin supplemented with superoxide dismutase (SOD, 60UI/mL) and GO-egg yolk-based extender, without antioxidant. Semen was cryopreserved and samples were analyzed by laboratorial tests such as computer assisted sperm analysis (CASA); plasma (eosin/nigrosin) and acrosome (fast green/ bengal rose) membrane integrity; mitochondrial cytochemical activity (DAB); DNA integrity (SCSA), and induced oxidative stress index (TBARS). Data were analyzed using the software Statistical Analysis System-SAS<sup>®</sup> (SAS, 2001), UNIVARIATE procedure was used and variables were tested by Barlett test, moreover, GLIMMIX procedure were used for variance analysis and for determining treatment meanings differences. The level of significance considered was 5%. It could be verified that the presence of antioxidant on semen extender did not protect sperm cells from oxidative stress (LA:640.36; LS:564.13; GO:255.23ng/10<sup>6</sup>sptz), and GO extender showed lower levels of TBARS. On the other hand, SOD was as efficient as egg yolk in preserving sperm cells with high mitochondrial potential (66.05%, 76.90%), respectively, in view of superoxide anion produced by mitochondria during the respiration process is catalyzed by SOD, protecting mitochondrial integrity from oxidative damages. In addition, AA exerted a greater protection on DNA induced denaturation, when compared to GO group (0.51%; 2.61%), respectively. Despite the protection of AA on DNA has been correlated, by several authors, to the capacity of reducing oxidative stress, it was verified that this protection involves mechanisms different from its antioxidant role. As it can be seen, soybean lecithin-based extender supplemented with SOD or AA would be an option for definitive replacement of egg yolk-based extender, avoiding sanitary barriers that prevent the international commercialization of bovine cryopreserved semen samples.



A018 Male Reproductive Physiology and Semen Technology

### **Comparison of the kinetics of sperm movement of boar semen subjected to room temperature or 37°C**

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**Keywords:** room temperature, sperm parameters, swine.

The aim of this study was to compare the influence of temperature on the quality of boar semen before dilution, by means of the computerized analysis of sperm kinetics. Were used ejaculates from four Large White boars, with an average age of 3 years, kept in a system of weekly semen collection. The samples were collected by the gloved hand technique with the aid of a dummy. Semen was collected in plastic tubes, protected by Styrofoam, prepared with sterile gauze on the top to separate and discard the gelatinous fraction of the ejaculate. After collection, the container was sealed and taken to the laboratory for evaluation. The ejaculate was aliquoted into two samples, one of which was kept in a Styrofoam container at room temperature (G1) and another was maintained at water bath at 37°C for 10 minutes (G2). The semen samples were evaluated for motility by CASA (Computer Assisted Sperm Analyzer, Hamilton-Thorne IVOS, Beverly, MA, USA) and further diluted 1:1 in 0.9% NaCl solution. The values of sperm velocity index and sperm movement index were calculated according to Núñez-Martínez, Moran and Peña (Reproduction in Domestic Animals, Volume 41, PP. 408-415, 2006). Sperm parameters for the two groups were tested for normality by the Shapiro-Wilk test followed by Mann-Whitney statistical method ( $P < 0.05$ ). The averages found for MOT and PROG MOT were respectively  $84.3 \pm 8.7$  and  $41.5 \pm 16.3$  for G1 and  $86 \pm 8.7$  and  $35.5 \pm 14.4$  for G2. The index sperm velocity and index movement sperm showed an average of  $210.9 \pm 38.2$  and  $165.5 \pm 17.4$  for G1 and averaged  $186.3 \pm 23.1$  and  $159.9 \pm 17$  for G2. There were no statistically significant differences between groups. It can be concluded that there are no differences in semen quality and sperm movement during storage in isothermal container or at 37°C. Thus, there is greater economy with the equipment to keep the semen at 37°C and more convenience in seminal evaluations at pig breeding farms.



A019 Male Reproductive Physiology and Semen Technology

### **Canine antioxidant profile of ejaculated and epididymal fluid**

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**Keywords:** antioxidants, dogs, epididymal fluid.

Epididymal maturation is a process that modifies sperm cells allowing them to fertilize properly, but these alterations increase sperm susceptibility to the reactive oxygen species attack (lipid peroxidation). As a mechanism of protection, the epididymis and the prostate must provide antioxidants in order to avoid the damage caused by the oxidative stress. So, this research aimed to compare the antioxidant enzymatic profile of the ejaculate and the distinct parts of the canine epididymis. We used five dogs of different breeds aged 1 to 6 years. From the same animal, the semen sample was obtained by digital manipulation and, after 1 week, from the epididymal segments (caput, corpus and tail), after orchietomy. The epididymis were stored at 5°C for up to 19 hours and samples were subsequently collected through small incisions (<1mm) in each segment, aspirated separately by automatic pipette and maintained in 100µl TALP extender. We evaluated the activity of catalase, glutathione peroxidase (GPx) and superoxide dismutase (SOD) in the three fractions of the ejaculate (fraction I, II and III) and in each epididymal segment. Quantification of catalase activity was measured by the consumption of H<sub>2</sub>O<sub>2</sub>, GPx activity was evaluated by the consumption of NADPH and SOD activity, by c-citocrome reduction by superoxide anion, using spectrophotometry. Data were compared by ANOVA and Tukey test ( $P \leq 0.05$ ) and submitted to Pearson and Spearman correlation test. There was no statistical difference regarding the concentration of SOD among samples from fraction I ( $80 \pm 35.6$  UI/mL), fraction II ( $116.1 \pm 17$  UI/mL) and fraction III ( $91.2 \pm 10.3$  UI/mL) of the ejaculate, as well as among samples from the epididymal caput ( $80.9 \pm 16.4$  UI/mL), body ( $102.2 \pm 19$  UI/mL) and tail ( $94.5 \pm 19.3$  UI/mL). For catalase, we obtained reduced enzymatic activity on fractions I ( $0.8 \pm 0.8$  UI/mL), II ( $1.7 \pm 0$  UI/mL) and III ( $2.9 \pm 1.8$  UI/mL), but we didn't find activity it in the epididymal segments. For the analysis of GPx, we found a higher activity in fraction II ( $45.5 \pm 15.7$  UI/mL), when compared to fractions I ( $15.5 \pm 2.2$  UI/mL) and III ( $8.7 \pm 1.7$  UI/mL) of the ejaculate. For the epididymis, the tail ( $80.6 \pm 9.2$  UI/mL) had superior concentration of GPx when compared to the body ( $49.4 \pm 7.2$  UI/mL) and caput ( $24 \pm 3.9$  UI/mL). The epididymal tail presented higher concentration of the GPx enzyme by having a higher protein secretion along sperm maturation. Moreover, we observed a positive correlation between SOD and GPx concentrations in the tail ( $r=0.86$ ,  $P=0.0012$ ) and body ( $r=0.79$ ,  $P=0.0064$ ) of the epididymis, which confirms the oxidative homeostasis against the lipidic peroxidation during sperm maturation through the epididymis. We can highlight the importance of GPx and SOD, which protects sperm during maturation. These enzymes must be considered in the composition of the extender for epididymal fluid used for reproductive biotechnologies in the canine species.



## A020 Male Reproductive Physiology and Semen Technology

### Assessment of sperm characteristics of rams treated with low-level laser therapy: preliminary results

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**Keywords:** insulation, laser therapy, rams, spermatozoa.

The low-level laser therapy (LLLT) is an important tool to induce cellular proliferation and to stimulate mitosis. This biostimulatory effect was observed in the seminiferous epithelium of rats treated with laser therapy (Taha and Valojerdi, 2004, *Lasers Surg. Med.*, 34, 4, 352-359). In this way, the aim of this study was to evaluate different LLLT protocols in testes of rams submitted to scrotal insulation for the induction of the testicular degeneration. For this, six rams were divided in three groups: 1) Control, without LLLT treatment (n=2); 2) LLLT treatment of cumulative dose of 28J/cm<sup>2</sup> (n=2); 3) LLLT treatment of cumulative dose of 56J/cm<sup>2</sup> (n=2). The treatment was performed once a day and repeated 48h later during 15 days in the treated groups (2 and 3). The output power used was the same for the treated groups (30mW). The scrotal insulation was done in all rams for 72h before the beginning of the treatment period. After 10 days of scrotal insulation, four semen collections were performed during the experimental period in intervals of 7 days each. The Sperm-Class Analyser (SCA; Microptic S.L., Barcelona, Spain) was used to the computer-assisted assessment of sperm motility. The differential interference-contrast microscopy (model 80i; Nikon, Tokyo, Japan) was used to assess sperm morphology. The epifluorescence microscopy (model 80i; Nikon, Tokyo, Japan; filter D/F/R, C58420, Nikon, Tokyo, Japan) was used to evaluate acrosomal membrane integrity (FITC-PSA), plasma membrane integrity (PI+Hoescht 33342) and mitochondrial function (JC-1). The data was analyzed employing the Statistical Analysis System (SAS Institute Inc., 1995). The treatment effect was tested for ANOVA, the repeated measures factor was added. The Tukey test was used to compare the averages when there was time X treatment interaction or time and treatment effect. There was no time X treatment interaction (P>0.05) for the variables analyzed. There was no difference in the total motility (TM, P=0.56), the progressive motility (PM, P=0.54) and the minor defects (MD; P=0.2) among the groups 1 (TM: 41.36±9.07, PM: 29.51±7.75%, MD:12.56±3.3%), 2 (TM: 60.11±9.60%, PM: 45.55±10.28%, MD: 8.56±2.22%) and 3 (TM: 58.03±7.24%, PM: 43.58±7.12%, MD: 10.43±1.74%). Treatment effect was observed only in acrosomal membrane integrity. The group 1 (85.53±2.08%) presented higher (P<0.05) percentage of acrosomal membrane integrity than group 2 (74.50±2.72%) and 3 (69.43±3.67%). There was time effect (P<0.05) to the plasma membrane integrity and mitochondrial function. Thus, it is concluded that LLLT no change the total and progressive motility, minor defects, plasma membrane integrity and mitochondrial function, but decreases the rate of sperm acrosome integrity. However, the results are preliminary and more studies are being done by our group.

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A021 Male Reproductive Physiology and Semen Technology

**Post-cryopreservation semen viability in bulls supplemented with protected fat and/or antioxidants**

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**Keywords:** fatty acids, selenium, spermatozoa.

Cryopreservation of semen is important for the expansion of reproductive techniques such as AI and *in vitro* fertilization; however, it induces oxidative stress in sperm cells. To enhance cell survival after freezing, the plasma membrane needs sufficient fluidity that is guaranteed by polyunsaturated fatty acids and antioxidant defense to protect against lipid peroxidation. The aim of this study was to evaluate post-thaw sperm characteristics from bulls fed a diet with or without rumen protected fat and / or antioxidants. A total of 48 Nelore bulls was confined (three animals per pen) and assigned to four treatments according to the addition of rumen protected fat and/or antioxidants to the diet. During 30 days (d), bulls were fed the same adaptation diet (sugar cane bagasse, citrus pulp, corn gluten meal, urea and mineral salt). Thereafter, for 75 d, the same diet was offered, differing in the addition of: F) fat protected from rumen degradation (rich in linoleic acid; Megalac-E<sup>®</sup>, 1.5% DM, n=12); A) antioxidant (EconomasE<sup>®</sup>, 3g/head/d, n=12); FA) Megalac-E and EconomasE (n=12); or C) control group (n=12). Semen collection and freezing were performed seven times: 0, 15, 30, 45, 55, 65 and 75 d after start of the experimental diet period. After thawing at 37°C for 30 s, semen analyses were performed by means of computerized analysis of sperm kinetics, flow cytometry for plasma membrane (PM) and acrosome membrane (AM) integrity, fluidity of PM and mitochondrial potential of spermatozoa. Evaluation of lipid peroxidation of sperm (Nichi et al., Theriogenology, 66, 822-28) was also done. Data were analyzed by repeated measures of GLIMMIX of SAS. Lipid peroxidation and the kinetics of thawed sperm were similar among diets (P>0.10). Moreover, diets without fat (C and A) increased the percentage of intact PM and unreacted AM (62.2±2.87 vs 53.3±2.87%, P<0.05). In addition, diets without antioxidants (C and F) caused an increase in the number of sperm cells with damaged PM and with induction of sperm *acrosome reaction*. After 65 d of supplementation, sperm of bulls fed diets with fat (F and FA) had higher PM stability, compared to the other treatments (60.4±2.62 vs. 52.7±2.62%, P=0.05), however there was a trend to increase the number of dead sperm. The mitochondrial potential of spermatozoa was not affected by treatments (P>0.10). A beneficial effect to the sperm cells after cryopreservation was found when bulls were supplemented with diets containing antioxidants, compared to diets containing fat.

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## A022 Male Reproductive Physiology and Semen Technology

### **Effects of shadow availability on reproductive traits of Nelore bulls**

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**Keywords:** artificial shading, Nelore, semen.

Bull is responsible for more than 90% of all pregnancies in herd, and its reproductive failure exerts much more impact on economy than reproductive failure coming from the bovine female. One of the main causes of bull reduction in reproductive performance is heat stress, which causes testicular degeneration (Hansen, 2004, *Animal Reproduction Science*, 82, 349-60). Also, Zebu bulls represent more than 80% of bovine males raised in Brazil. This study aimed to evaluate the effect of thermal stress reduction, through shade availability, on reproductive characteristics of Nelore bulls (*Bos indicus*). For this, ten bulls were divided in two experimental groups: available artificial shade (CS, n=5) and unavailable shade (SS, n=5). Each group was kept in two-hectare paddocks, in which shade availability for group CS was artificially created, using an area with poliethylen Sombrite (Polysack®), with 90% of solar light retention and a total of 10 m<sup>2</sup> of shade per animal. In SS group, animals were maintained in paddock without shadow access. Animals were submitted to semen collection every 15 days, for four months. Samples were evaluated according to laboratory standards, with a computer assisted sperm analysis (CASA), morphology (DIC) and sperm viability (fluorescent probes: IP, FITC-PSA and JC1). Data were analyzed using ASA statistical software SAS (2004). Statistical analysis included the factor of repeating measures in time, referring to several times of the sampling. The probability of interaction with the time was determined by the Greenhouse-Geisse test, using the REPEATED command generated by the GLM proceeding (PROC GLM of the SAS). Level of significance considered was 5%. No interaction was observed between treatments (CS and SS) and time (8 collections) for all analyzed variables ( $P>0.05$ ). No significant effects of treatment were observed (CS vs SS) for studied variables such as: progressive motility ( $77.7 \pm 1.3$  vs  $79.7 \pm 1.2$ ,  $P=0.35$ ), morphology ( $23.7 \pm 2.3$  vs  $16.7 \pm 1.2$ ,  $P=0.28$ ) and sperm viability ( $66.8 \pm 1.7$  vs  $70.9 \pm 1.2$ ,  $P=0.64$ ). So, it can be concluded that the absence of shaded areas, during summer, does not negatively affect reproductive characteristics in Nelore bulls raised in the Southeast of Brazil. These results also suggest that, even in an adverse condition of ambient temperature, Zebu bulls were able to maintain all thermoregulation mechanisms.



A023 Male Reproductive Physiology and Semen Technology

**Effect of seminal plasma and egg yolk concentration on freezability of buck semen**

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**Keywords:** centrifugation, extender, male goat.

Goat semen has a feature to be considered for cryopreservation because the seminal plasma interaction with egg yolk may be detrimental to sperm (Roy, 1957, *Nature*, 179, 318-319; Gibbons, 2002, *Revista Taurus*, 16, 24-32). Therefore, the aim of this work was to evaluate, *in vitro*, the effects of seminal plasma and egg yolk on the viability of cryopreserved goat semen. The extender used was the yolk citrate, where it was split into two equal aliquots: one of the samples was added 5% egg yolk (2.5 mL egg yolk: 47.5 mL citrate solution) and another was added 10% egg yolk (5.0 mL egg yolk: 45 mL citrate solution). Semen was cryopreserved using traditional refrigerator and liquid nitrogen vapor (N<sub>2</sub>L). After a minimum of 24 hours in N<sub>2</sub>L, the straws were thawed. Sperm motility and vigor after thawing and thermo resistance test (TRT) were evaluated. Data were submitted to analysis of variance and means compared by the F test, at 5.0% significance level. The observed values for motility and vigor after thawing and fast and slow thermo resistance test (TRT), according to the presence of seminal plasma and egg yolk percentage, were: 5% egg yolk with plasma (25.0% and 3.3; 1.60% and 0.7; 12.36% and 1.6, respectively); 5% egg yolk without plasma (23.61% and 3.1; 1.25% and 0.2; 9.93% and 1.3, respectively); 10% egg yolk with plasma (30.8% and 3.3; 4.4% and 1.9; 19.5% and 2.7, respectively); and 10% egg yolk without plasma (13.4% and 2.5; 4.1% and 0.5; 17.0% and 1.0, respectively). There were significant differences between the analyzed data (P <0.05) as related to semen with or without plasma, with different percentages of egg yolk. The group presenting the best results was 10% egg yolk citrate in extender with plasma. These results may have occurred because of the seminal plasma with higher concentration of egg yolk promotes greater protection to sperm during cryopreservation. This combination stabilizes the plasma membrane and reduces its disruption in the freezing step, by decreasing the activity of oxygen reactive species, which can cause damage to sperm DNA (Azerêdo, 2001, *Small Ruminant Research*, 41, 257-63). The positive results for the presence of plasma can be explained by the fact that the work was conducted in the breeding season, where levels of phospholipase A are lower as compared to the non breeding season. Also, we used young animals (10-month to 1-year old), which are still in the process of development of the reproductive system. These animals have lower phospholipase A compared to adults, explaining the results obtained in this study. Thus, we conclude that the presence of seminal plasma and higher concentration of yolk extender provided greater viability of cryopreserved goat semen.



A024 Male Reproductive Physiology and Semen Technology

**Glycerol and dimethylacetamide association for the ovine sperm cryopreservation**

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**Keywords:** cryoprotectants, ovine, sperm cryopreservation.

Ten semen samples of five Santa Ines rams were cryopreserved with the objective of analyzing the use of cryoprotectants glycerol (GL) at 6% (GL6%) and dimethylacetamide (DMA) at 3% (GL3%) or in different levels of association of both (GL5%+DMA1%, GL4%+DMA2%, GL3%+DMA3%, GL2%+DMA4% e GL1%+DMA5%). The base extender was Tris-egg yolk. Right after evaluation, semen was diluted in the different extenders, cooled to 5°C and samples were frozen in liquid nitrogen vapor. After thawing, the kinetic sperm parameters were analyzed (total motility-TM, progressive motility-PM and sperm vigor-VIG). Aliquots were collected for the supravital test with dye eosin (EOS); the sperm morphology was analyzed and the percentage of bent tails (BT) calculated. The functional integrity of the plasmatic membrane was studied by using osmotic shock (OS) to which part of the semen was added to 10 parts of the final solution with deionized water. After the diluted semen incubation for five minutes at 37°C, it was fixed with 10 µL of formaldehyde buffered saline. The percentage of reactive spermatozoa to the OS was determined by subtracting the percentage of spermatozoa with OS-induced BT, from the BT obtained after thawing. These evaluations were carried out in phase contrast microscopy (1000x) and one hundred cells were analyzed per semen sample. All the statistical analysis were performed by using the SAS software, version 5.0 (1996) (MEANS and GLM Procedure with p<0.05). The averages (%) of post-thaw total motility, viability by the supravital and osmotic shock were as follow: GL6%: 66.0; 36.2 and 53.2; DMA3%: 40.0; 46.2 and 33.2; GL5%+DMA1%: 69.0; 40.4 and 42.2; GL4%+DMA2%: 69.0; 33.6 and 29.4; GL3%+DMA3%: 60.0; 40.2 and 28.6; GL2%+DMA4%: 49.0; 39.6 and 29.8 and GL1%+DMA5%: 43.0; 33.0 and 32.0. We observed the greatest (P<0.05) rates of total motility for GL6%, GL5%+DMA1% and GL4%+DMA2% extenders in relation to the group with higher level of dimethylacetamide (GL1%+DMA5%) or when the dimethylacetamide was used alone (DMA3%). Despite the large numerical variations, the rates of EOS, OS and BT did not differ (P>0.05) among the groups. It can be concluded that the dimethylacetamide and glycerol association was effective for maintenance of sperm viability. Therefore, dimethylacetam.

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A025 Male Reproductive Physiology and Semen Technology

**Are DNA fragmentation, sperm viability and oxidative stress correlated in rams?**

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**Keywords:** DNA fragmentation, ovine, sperm.

Sperm DNA integrity is essential for embryonic development (Fatehi et al., 2006, *Journal of Andrology* 27, 176-88). Protamination damage, apoptosis and oxidative stress can cause changes in sperm DNA (Tsakmakidis, 2010, *Small Ruminant Research*, 92, 126-30), acting alone or in combination. Changes in chromatin lead to a more vulnerable sperm, which could turn on the apoptotic process and generation of reactive oxygen species. Little information is known about sperm DNA fragmentation in rams. The aim of this study was to investigate correlations between changes in DNA sperm, oxidative stress and sperm viability in rams. We used 12 rams to collect semen once a week during 9 weeks by artificial vagina. To study seminal viability, we conducted the following analysis: sperm motility, vigor, mass motility, concentration, and morphology; integrity of plasma and acrosomal membranes (propidium iodide / FITC-PSA) and mitochondrial membrane potential (JC1) by flow cytometry. To evaluate sperm DNA damage, the sperm chromatin structure susceptibility assay and alkaline Comet were used. Oxidative stress was measured by lipid peroxidation, using spontaneous and induced TBARS, and intracellular hydrogen peroxide was stained by dichlorofluorescein. Spearman correlation was determined between the dependent variables and significant correlations was considered when  $p < 0.05$ . In the ram semen, sperm motility, vigor and mass motility are highly correlated ( $0.75 < r < 0.86$ ). These variable are inversely correlated with morphological changes ( $-0.34 < r < -0.41$ ), mitochondrial activity ( $-0.19 < r < -0.25$ ) and lipid peroxidation ( $-0.17 < r < -0.31$ ). In addition, the presence of intracellular free radicals were correlated with low mass motility ( $r = -0.18$ ). The greater the percentage of sperm with fragmented chromatin, the greater the morphological defects ( $r = 0.18$ ) and spontaneous lipid peroxidation ( $r = 0.19$ ); and lower values of sperm motility ( $r = -0.22$ ), vigor ( $r = -0.18$ ), mass motility ( $r = -0.22$ ) and integrity of plasma and acrosomal membranes ( $r = -0.26$ ). Finally, higher DNA fragmentation was associated with higher susceptibility to oxidative stress, as measured by the increase in induced lipid peroxidation ( $r = 0.19$ ). In conclusion, there are significant correlations among sperm viability, oxidative stress and DNA fragmentation, given that the magnitude and significance of such correlations depend on the studied parameter and analytical method. Thus, we suggest that a cause and effect relation exists among those criteria or actions of common factors are involved in the physiological events during spermatogenesis.



A026 Male Reproductive Physiology and Semen Technology

**Pregnancy rate in Santa Inês ewes inseminated with different sperm concentrations**

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**Keywords:** insemination, ovine, sperm concentration.

The objective of the present study was to evaluate pregnancy rates of Santa Inês ewes inseminated with low concentrations of sperm cells. This work was conducted in the city of Esplanada, in the state of Bahia. We used 32 Santa Inês sheep divided into groups according to the concentrations of insemination doses. The animals were divided into four groups containing 8 animals each, named G90, G60, G30, and G15, with 90, 60, 30, and 15 million viable sperm cells, respectively. The sheep were subjected to a synchronization protocol, as follows: on D0, the progesterone implant was inserted (Progespon<sup>®</sup>, Schering-Plough Intervet), on D6, applied 300UI eCG (Novormon<sup>®</sup>, Syntex) and 0.5 mL of prostaglandin (Ciosin<sup>®</sup>, Schering-Plough) were injected; on D7, the implant was removed, on D8, 300UI HCG was given to the animals (Vetecor<sup>®</sup>, Hertape Calier). Artificial insemination occurred on D9, laparoscopically, between 52 and 56 hours after removal of progesterone implant. Pregnancy diagnosis was performed 30 days after AI via transrectal ultrasound, with a frequency of 5 MHz. A statistical analysis was performed using the Tukey test for ANOVA, with PROC GLM, in SAS 8, with significance levels of  $p < 0,05$ . Pregnancy rates for G90, G60, G30 and G15 were 50%, 37.5%, 25% and 25%, respectively, showing no statistical differences ( $P > 0.05$ ) between groups. These results are consistent with studies previously presented, demonstrating that the use of low sperm concentration can be applied. However, further studies are needed to seek its improvement, since it generates savings because of increased doses per ejaculate.



A027 Folliculogenesis, Oogenesis and Superovulation

### **Antral follicle counts in Nellore females with different reproductive parameters and body condition score**

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**Keywords:** antral follicular population, *Bos taurus indicus*, physiological status.

This study aimed to determine the effect of some reproductive parameters and Body Condition Score (BCS) on Antral Follicular Counts (AFC) in Nellore females. For this purpose, 481 Nellore females submitted to a protocol for follicular wave synchronization (Ferraz et al. 2010, *Acta scientiae veterinariae* 38, 728) were used. On D4, females were examined by transrectal ultrasonography using a 8MHz linear transducer (Pie-Medical, 100 Falco, Maastricht, Holanda) to determine the population of antral follicles  $\geq 3$ mm and the Ovarian Diameter (OVD). In this study, the parameters evaluated were animal category (CAT), postpartum interval (PPI), OVD and BCS. Within CAT, animals were classified as lactating cows (LC, n = 313), dry cows (DC, n = 70) and heifers (HEI, n = 98). Within PPI, DC were divided into PPI1 (n=28), PPI2 (n=101) and PPI3 (n=160), for animals at  $\leq 63$ , between 63 and 105 and  $\geq 105$  days postpartum, respectively. Ovaries of 216 females were measured and those with diameters  $\leq 1.90$ , between 1.90 and 2.50 and  $\geq 2.50$ cm were grouped into OVD1 (n = 50), OVD2 (n = 73) and OVD3 (n = 93), respectively. Finally, BCS of 311 animals was evaluated considering the scale from 1 to 5, and those with BCS  $\leq 2$ , between 2 and 3 and  $\geq 3$  were classified as low (n=42), intermediate (n=125) and high BCS (n=144). The data were analyzed by the ANOVA and Pearson correlation, with  $P < 0.05$ . The overall mean AFC was  $46.52 \pm 22.47$  follicles (FOL), showing a high variability for this trait, ranging from 7 to 145 FOL/animal. AFC did not vary between DC ( $47.00 \pm 23.50$  FOL), LC ( $46.55 \pm 23.08$  FOL) and HEI ( $45.17 \pm 19.37$  FOL). AFC did not vary between PPI1 ( $41.39 \pm 16.14$  FOL), PPI2 ( $49.06 \pm 22.94$  FOL) and PPI3 ( $48.66 \pm 25.31$  FOL) either. BCS did not alter AFC, which were  $45.17 \pm 27.35$ ;  $44.60 \pm 21.95$  and  $45.54 \pm 22.48$  FOL for animals with low, intermediate and high BCS, respectively. However, OVD had a significant impact on AFC, which were  $32.24 \pm 13.99$ ;  $44.53 \pm 17.66$  and  $57.70 \pm 23.96$  FOL for animals at OVD1, OVD2 and OVD3, respectively. In addition, there was a high positive correlation ( $r=0.53$ ;  $P=0.0003$ ) between OVD and AFC. The present results suggest that reproductive status, postpartum interval and body condition do not seem to affect AFC. The positive relationship between OVD and AFC may allow the use of OVD as a parameter to estimate AFC in Nellore females.



A028 Folliculogenesis, Oogenesis and Superovulation

### Effects of levels of sulfur and cobalt on the diet on biochemical parameters, gene expression, and viability of bovine oocytes

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**Keywords:** epigenetic reprogramming, nutrition, s-adenosylmethionine.

S-adenosylmethionine, produced from methionine, is a precursor of methyl groups for the establishment of DNA methylation. Methionine comes from the diet and remethylation of homocysteine or betaine, which derives from choline. It is necessary to reprogram DNA methylation to form oocytes and embryos, and methylation is susceptible to nutrition changes. We evaluated if sulphur and cobalt deficiency would affect the levels of blood biochemical components of the methionine cycle and metabolism of glucose, gene expression, quality of oocytes and embryo production rate. Thirty Nellore pubertal heifers (10/treatment) submitted to ad libitum diet offered twice a day for 5 months were divided in 3 groups: sulphur+cobalt-deficient group: sugar cane and urea - sulphur (1%) and mineral premix - cobalt and sulphur (100g/animal), control group: sugar cane and urea (1%) + sulphur (9:1) and complete mineral premix (100g/animal); the methionine+choline group: the same diet of the control group, + salt (100g/animal) encapsulated (Kemin<sup>®</sup>) methionine (50%; 20g/animal) and choline (50%; 15g/animal). B9 and B12 vitamins, homocysteine, insulin, IGF-I and glucose levels were measured in blood plasma each 15 days during the dietary period. Animals were submitted to OPU weekly since the third month of the diet. Oocytes in stages I, II and III were considered viable. Part of the oocytes was denuded, and expression of MAT2B, mSHMT2, SAHH, DHFR, DNMT1 and MTR and the constitutive genes GAPDH and  $\beta$ ACTINA was evaluated in cumulus cells. Remaining oocytes were used for *in vitro* embryo production. Data were evaluated by Student's t test or Mann-Whitney test. The average consumption of salt, choline and methionine was 19.5 kg 93.1 g, 18.6 g and 14 g; 21 kg, 97 g, 0.0 g and 0.0 g; 20 kg, 95.5 g 0,0 g and 0.0 g for the methionine+choline group, control and the sulphur+cobalt-deficient group, respectively. The sulphur+cobalt-deficient group differed from the control group for homocysteine (13.8 $\pm$ 5.69 vs 10.6 $\pm$ 3.19; P=0.0001), B9 (29.9 $\pm$ 11.94 vs 26 $\pm$ 10.52; P=0.0156); B12 (143.8 $\pm$ 30.07 vs 165.6 $\pm$ 48.7; P=0.0001) IGF-I (375.3 $\pm$ 95.6 vs 410.2 $\pm$ 121; P=0.024) and glucose (82.2 $\pm$ 18.5 vs 76.2 $\pm$ 13.9; P=0.0142). Among the genes evaluated, MAT2B ( $\beta$ actina P=0.0636; GAPDH: P=0.0565) and DNMT1 ( $\beta$ actina P=0.0105; GAPDH: P=0.032) expression was reduced in the sulphur+cobalt-deficient group in relation to the control group. The methionine+choline group (46.0 $\pm$ 8.78%) compared to the control group (60.5 $\pm$ 8.76%), presented a lower rate of viable oocytes (P=0.0285), with no difference for blastocyst (D7) and hatching rates (D8). The alteration of dietary compounds affected the level of metabolites, the pattern of gene expression and morphological quality of the oocytes. Whether there were alterations in epigenetic reprogramming of the oocyte capable of affecting the viability of the embryos without changing embryo production rates remains to be investigated.

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A029 Folliculogenesis, Oogenesis and Superovulation

### **Effect of enalapril on pregnancy rate in goats submitted to fixed-time artificial insemination**

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**Keywords:** angiotensin-converting enzyme, caprine, ovulation.

Enalapril, an angiotensin-converting enzyme inhibitor, increased the efficiency of a protocol for fixed-time artificial insemination (TAI) in goats, when applied during the eleven days of the estrus synchronization protocol. However, there is a need to simplify the protocol, to provide convenience to its use in farm conditions. The aim of this study was to evaluate the effect of ACE inhibition on pregnancy rates in goats subjected to TAI associated with the use of a single dose of enalapril in a slow-release vehicle by two routes of administration. A total of 94 goats were subjected to estrus synchronization with intravaginal sponges (Progespon<sup>®</sup>, Syntex, Argentina) impregnated with 60 mg of medroxyprogesterone acetate-MAP inserted on day zero (D0) for a period of 12 days followed by intramuscular injection of 300 IU of Equine Chorionic Gonadotropin (Novormon<sup>®</sup>, Sintex, Argentina) and 75 µg of cloprostenol (Prolise<sup>®</sup>, Tecnopec, Brazil) on the tenth day of treatment (D10). Three groups were formed: group 1 (G1; n = 34) control, G2 (n = 30) received vaginal ovules containing 60 mg of enalapril maleate on D10, and G3 (n = 30) received 3 ml of an oily enalapril maleate suspension at 20 mg/mL subcutaneously on D10. There were two inseminations with fresh semen, from males of proven fertility, diluted in coconut water the first was done 36 hours after sponge removal (dose = 0.5 mL) and the second 12 hours after the first insemination (dose = 0.25 ml). Pregnancy diagnosis was performed by transrectal ultrasonography 35 days later. Data were analyzed by  $\chi^2$  test ( $p < 0.05$ ). The overall pregnancy rate was 60.63% (57/94), while it was 61.76% (21/34) in the control group (G1), 63.33% (19/30) in the intravaginal enalapril group (G2) and 56.66% (17/30) in subcutaneous enalapril group (G3). There was no significant difference between groups. The results show that treatment with enalapril in a single dose was not sufficient to increase pregnancy rates. Other experiments are required to determine the minimum number of days necessary to obtain the increase previously observed in the treatment of eleven days.



A030 Folliculogenesis, Oogenesis and Superovulation

### **Estimate of number of antral follicles in Nelore females in reproductive age**

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**Keywords:** *Bos indicus*, follicles, population.

The estimate of the number of antral follicles in the ovary has been investigated by many research groups in the world. The relation between number of follicles and hormone levels was demonstrated as a possible parameter to represent the reproductive potential in *Bos Taurus* females (Ireland et al., 2011, *Reprod. Fertil. Dev.* 23, 1–14). The objective of this study was to determine the population of antral follicles in Nelore females. Were used, during the month of October of 2012, 204 Nelore females with body condition score between 2.25 and 3.5 (scale 1-5), including prepubertal heifers (n = 62), pubertal heifers (n= 36), primiparous cows (n = 32) and multiparous cows (n = 73) with 30 to 45 days postpartum. The ages of the animals ranged from 22 to 24, 22 to 26, 36 to 40 and from 48 to 98 months, respectively, in the four categories mentioned above. Animals were kept in pasture of *Brachiaria brizantha* with mineral supplementation *ad libitum* containing 9% phosphorus. All animals were submitted to two transrectal ultrasonographic examinations (7.5 MHz transducer, Mindray, China), with an interval of 10 days, to assess cyclicity by identifying a corpus luteum in one of two exams and to count all antral follicles (AFC). Data were submitted to nonparametric analysis of variance (P < 0.05). Mean AFC values were similar in all groups (p > 0.05), and were 16, 18, 20 and 18 for prepubertal heifers, pubertal heifers, primiparous cows and pluriparous cows, respectively. Data were grouped, and females with AFC up to 18 follicles were classified as intermediate AFC and with more than 18 follicles were classified as high AFC. The distribution of females with high AFC was 40% (25/62), 55.5% (20/36) for pre-pubertal and pubertal heifers and 56.2% (18/32) and 45.2% (33 / 73) for primiparous and pluriparous cows, respectively. There were differences (P < 0.001) in AFC values in each category (Intermediate AFC: 14, 16, 14:14 vs. High AFC: 24, 22, 23, 24), and similarity (p > 0.05) in intermediate and high AFC values between categories, respectively for pre-pubertal and pubertal heifers and primiparous and pluriparous cows. This study suggests that AFC in the ovaries of Nelore females in reproductive age is similar, and lower numbers (20-25) compared to the results previously published in Nelore females were observed, possibly because animals from commercial herds and evaluated by transrectal ultrasonography were used.



A031 Folliculogenesis, Oogenesis and Superovulation

### **Estimate of ovarian follicular population in mules: preliminary results**

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**Keywords:** follicles, mule, ovarian.

Female mules are classically considered infertile, although some mules are able to manifest estrus at regular intervals. Due to the shortage of embryo recipients in the equine and asinine species, as well as to the increased number of mules in our country, this paper proposes an investigation of one reproductive aspect of mules, the ovarian follicular population. During the months of March and April of 2013, 3 ovaries of mules were collected, with no information regarding age or the respective pairs of ovaries, being randomly selected. The ovaries were collected from a slaughterhouse, which did not allow further information on these animals. For an hour, the ovaries were transported to the laboratory at 20 ° C, where they were measured and weighed. Each ovary was fragmented according to its size, being one ovary divided into 8 fragments and the other 2 ovaries divided into 2 fragments. Then, the ovaries were fixed in a Bouin solution for 24 hours and kept in 70% ethanol. Later, they were dehydrated in increasing concentrations of alcohol, diaphanized in xylene and infiltrated in paraffin. With the inclusion of paraffin, serial sections of 0.5 micrometers were performed, with a rotary microtome (Leica ®, Wetzlar - Germany). For each 50<sup>th</sup> section, a histological slide was assembled. The staining was performed with periodic acid-Schiff (PAS) and hematoxylin for structural analysis in microscopy. We analyzed 260 blades, from which only 6 disposed follicles located in small clusters. Until now, mixed results were found regarding the parameters analyzed: in one ovary, with the weight / volume of 9.17 g / 9 mL, 22 follicles were observed, of which 21 were primary and 1 was antral. In the second ovary (2.3 g / 2 ml), no follicles were found; and in the third ovary (1.61 g/2mL), only 3 primary follicles were present. The ovarian parenchyma was quite distinct from the equine and asinine species; with significant vascularization, macroscopically visible. Considerable variation in weight and size were noted, as well as in the number of follicles. The variation can be explained by the great reproductive variability, including follicular reserve by mules, because the species of origin (equine and asinine) have different numbers of chromosomes. There is a big gap in the morpho-physiological characteristics of mule ovaries, due to the extreme difficulty in obtaining ovaries of these animals. By the initial data obtained in this study, quite unique particularities were found regarding the organization of the parenchyma and vascular distribution, in addition to follicular population. These findings may be related to variations in the fertility found in female mules.



A032 Folliculogenesis, Oogenesis and Superovulation

### **Fibroblast growth factor 10 (FGF10) inhibits expression of genes that regulate steroidogenic and ovulatory capacity in bovine granulosa cells**

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**Keywords:** cattle, FGF10, follicle.

Fibroblast growth factors are grouped into subfamilies in accordance with structural and binding properties. FGF10 belongs to the FGF7 subfamily and activates receptors FGFR2B and FGFR1B. In bovine antral follicles, FGF10 expression was detected in oocytes and theca cells, where mRNA levels were negatively correlated with estradiol intrafollicular concentrations (Buratini et al., 2007, *Mol Reprod and Dev*, 77, 743-750). FGF10 inhibited estradiol production in cultured granulosa cells (GC) and blocked follicle development when administered via intrafollicular injections (Buratini et al., 2007, *Mol Reprod and Dev*, 77, 743-750; Gasperin *et al.*, 2012, *Reproduction*, 143, 815-823.). Moreover, FGF10 and FGFR2B mRNA expression was lower in dominant compared to subordinate follicles around follicle deviation. To gain more insight into the mechanisms by which FGF10 inhibits follicle development, the effects of FGF10 on mRNA abundance of CYP19A1, HSD3B1, HSD17B, CYP11A1, STAR, LHR, MVK (mevalonate kinase), FSHR, IGFBP4, IGFR1, IGFR2, PAPP, AGTR2 and GADD45 in cultured bovine GC were assessed. Small antral follicles (2-5mm) were dissected from abattoir ovaries, GC were separated and cultured in serum-free medium with graded doses of recombinant FGF10 (0, 1, 10 and 100 ng/ml; R&D Systems®) for six days with partial medium change (70%) every two days. GC were recovered and submitted to total RNA extraction. Effects of FGF10 on gene expression were assessed by real time RT-qPCR using bovine-specific primers and PPIA as the endogenous control. Relative expression was determined by the Pfaffl's equation. Effects of FGF10 treatment on mRNA expression was tested by ANOVA and means were compared using the Tukey-Kramer HSD test. Significant differences were considered when  $P < 0.05$ . FGF10 decreased mRNA abundance of CYP19 ( $P=0.02$ ; at 100 ng/ml), FSHR ( $P=0.0001$ ; at 1 ng/ml), IGFR1 ( $P=0.04$ ; at 1 ng/ml), AGTR2 ( $P=0.02$ ; at 10 ng/ml) and MVK ( $P=0.03$ ; at 10 ng/ml). In conclusion, the present data suggest that suppression of aromatization capacity and FSH, IGF and AngII signaling are included in the mechanisms by which FGF10 inhibits antral follicle development.

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A033 Folliculogenesis, Oogenesis and Superovulation

### **Ovarian response following the use of estradiol benzoate or estradiol cypionate as inductors of ovulation in TAI synchronization protocols in Holstein cows**

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**Keywords:** estrogen, inductor, ovulation.

The objective of the present study was to evaluate the use of Estradiol Benzoate (EB) or Estradiol Cypionate (EC) as inductors of ovulation in Timed Artificial Insemination (TAI) protocol in Holstein females. A total of 50 females (31 cows and 19 heifers) that presented a body condition score of  $3.04 \pm 0.04$  (1 to 5 scale) at the first day of the synchronization protocol were used. At the first day (D0), they received an intravaginal progesterone device [P4; PRIMER®, Agener União; new device for cows and; used device for heifers (8 days)] associated with a 2mg i.m. injection of EB (Sincrodiol®, Ourofino Saúde Animal). At D8, the devices were removed and it was administrated i.m. 500µg of sodium cloprostenol (Ciosin®, MSD Saúde Animal). At this time, animals were randomly allocated into one of two different treatments, according to the inductor of ovulation utilized: EC at D8 (1,0mg i.m. of ECP®, Zoetis Brasil; n=25) and EB at D9 (n=25). Ultrasonographic exams (IBEX PRO, E.I. Medical Imaging, EUA) were realized every 24 hours between D8 and D10 and every 12 hours between D10 and D12. At D17, the volume of the formed corpus luteum was also measured. The statistical analysis was performed by the GLIMMIX procedure of SAS. There was no difference between the inductors of ovulation (EB vs. EC) according to the diameter of the follicle at D8 ( $10.9 \pm 0.6$  vs.  $12.2 \pm 0.6$ ;  $P = 0.13$ ), estrus occurrence (84% vs. 88%;  $P = 0.98$ ) and time of estrus between D8 and D10 ( $72.5 \pm 1.2$  vs.  $71.5 \pm 2.1$ ;  $P = 0.29$ ), diameter of the ovulatory follicle ( $13.9 \pm 0.5$  vs.  $15.0 \pm 0.5$ ;  $P = 0.10$ ), ovulation rate (92% vs. 92%;  $P = 0.94$ ), time of ovulation related to D8 ( $72.5 \pm 1.2$  vs.  $71.5 \pm 2.1$ ;  $P = 0.29$ ) and volume of CL at D17 ( $7,199.29 \pm 678.39$  vs.  $7,441.68 \pm 672.21$ ;  $P = 0.95$ ). However, it was observed that the EC determined more dispersed ovulations ( $P = 0.04$ ) than females treated with EB. Such greater variation in timing of ovulation was mainly related to the higher ( $P = 0.06$ ) occurrence of early ovulation ( $\leq 60$  hours) observed in those females treated with EC (28%) than in females treated with EB (8%). Thus, although the higher dispersion of the timing of ovulation observed in the EC treated animals than those treated with EB, both inductors of ovulations resulted in a satisfactory ovarian response in synchronization of ovulation protocols for TAI in Holstein females.



A034 Folliculogenesis, Oogenesis and Superovulation

### **Plasma and intrafollicular testosterone concentration in Nelore and Angus heifers with high and low follicle counts**

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**Keywords:** bovine, follicle, testosterone.

During follicle development androgens are synthesized by theca cells under the influence of LH through the conversion of androgen precursors. In monkeys and mice, androgens act in the follicle promoting the expression of growth factors (IGF1, GDF9, TGFB) and FSH receptor (Vendola et al., 1999, Biol Reprod 61, 353-7; Dumesic e Abbott, 2008, Semin Reprod Med 26, 53-61). In previous studies with these same animals we have shown that follicles from Angus heifers had higher expression of *IGF1R*, *IGF2* and *FSHR* (Favoreto et al., 2013, Reprod Fert Dev 25, 237 e Ereno et al., 2013, Reprod Fert Dev 25, 236). This experiment was designed to evaluate plasma and intra-follicular testosterone concentrations in Nelore and Angus heifers with high (HFC) and low (LFC) follicle counts. Sixteen Nelore and nineteen Angus heifers (24 months approximately) were kept in *Brachiaria bizantus* grass, animals also received a mix of grains and had access to salt and water ad libitum. Estrus was synchronized with two doses of PGF2 $\alpha$  11 days apart. To determine the number of follicles for each animal three ultrasound (US) exams were performed on day 1 of subsequent cycles. Animals were slaughtered 24 h after ovulation. Blood was collected before slaughter. Follicular fluid was collected from three follicles with 2-4 mm diameter. Plasma and intrafollicular testosterone concentrations were determined using the enzyme-linked immunosorbent assay specific for bovine testosterone (USCN Life Sciences, Wuhan, China). Data were analyzed using the PROC GLM and PDIF from SAS (SAS 9.2), considering the effects of breed and group (HFC and LFC). Plasma concentration of testosterone was higher ( $P < 0.001$ ) in Angus heifers ( $6.8 \pm 0.8$  ng/ml) when compared with Nelore heifers ( $1.5 \pm 0.9$  ng/ml), however there was no difference between groups within breed. Intrafollicular concentration of testosterone was also higher ( $P < 0.02$ ) in Angus heifers ( $46.0 \pm 4.6$  ng/ml) when compared with Nelore heifers ( $29.2 \pm 4.9$  ng/ml), but no difference between groups within breeds was found. In conclusion, higher plasma and intrafollicular testosterone concentration in Angus heifers might account for higher expression of growth factors and *FSHR* in follicles from these animals. However, testosterone is not involved in follicle recruitment.

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A035 Folliculogenesis, Oogenesis and Superovulation

### **Influence of ascorbic acid on *in vitro* culture of equine preantral follicles: preliminary results**

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**Keywords:** ascorbic acid; equine preantral follicles; *in vitro* culture.

The equine ovary has important features that hinder its handling, as the differentiated disposal of the cortical (internal) and medullar (external) areas and the sparse population of preantral follicles. Moreover, the difficulty in obtaining ovaries has led to a lack of information regarding folliculogenesis in this species. The aim of this study was to evaluate the effect of adding different concentrations of ascorbic acid in the *in vitro* culture of preantral follicles in horses. All ovaries were obtained from a local slaughterhouse. The ovaries (n = 10) of five mares were collected, washed in 70% ethanol and PBS. The internal region (cortex) was divided into nine fragments of approximately 6x6x2 mm. One fragment was immediately fixed in Bouin (control) and the others were transported to the laboratory in PBS with antibiotics at 4°C. In the laboratory, they were cultured at 39°C in atmosphere with 5% CO<sub>2</sub> for 2 to 6 days, with 2 ml of Minimum Essential Medium (MEM) supplemented with ITS (Insulin-Transferrin-Selenium), pyruvate, glutamine, hypoxanthine, bovine serum albumin and antibiotics (MEM+) or MEM+ added with ascorbic acid (25, 50 or 100 µg/ml). The medium exchange was performed every two days. After the culture period, the ovarian fragments were fixed in Bouin and processed for histology. Preantral follicles were classified according to the stage of development as primordial and developing follicles (primary and secondary), and according to viability in normal or degenerated. One hundred and sixty-two follicles were evaluated in the control group, 79 (D2) and 33 (D6) in MEM, 89 (D2) and 33 (D6) in 25 µg/ml, 143 (D2) and 9 (D6) in 50 µg/ml, and 96 (D2) and 4 (D6) in 100 µg/ml, totalizing 649 follicles. The results showed that, compared to non-cultivated tissue (control), the culture of preantral follicles *in situ* reduced the percentage of normal follicles in all the tested media (P < 0.05). In all treatments, there was a reduction in the percentage of primordial follicles, with a concomitant increase in the percentage of developing follicles when compared with control (88.9% primary, 11.1% developing follicles), and the highest percentage of developing follicles (77.1%) was obtained with the concentration of 50 µg/ml of ascorbic acid. In conclusion, 50 µg/ml of ascorbic acid was effective in promoting the activation of primordial follicles in horses.



A036 Folliculogenesis, Oogenesis and Superovulation

### **Influence of the ovarian reserve on the numbers of antral follicles in *Bos indicus* and *Bos taurus* purebred cows**

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**Keywords:** antral follicular count; bovine, preantral follicles.

The aim of the present study was to compare the population of preantral ovarian follicles among *Bos indicus* and *Bos taurus* purebred females with high and low numbers of antral follicles during follicular waves. Ovaries from Nelore (*Bos indicus*, n = 100) and Aberdeen Angus (*Bos taurus*, n = 100) cows (72-96 months-old) were collected at abattoirs and transported to the laboratory. After collection, ovaries were evaluated by ultrasonography and antral follicles  $\geq 3$  mm were counted using a microconvex array. Cows were assigned into groups with high (G-High) or low (G-Low) antral follicle count (AFC) based on the mean number of antral follicles  $\pm$  SD (Nelore, mean = 39 follicles; Angus, mean = 29 follicles) as follows: *Bos indicus* cows with high- ( $\geq 57$  follicles, n = 8) or low-AFC ( $\leq 21$  follicles, n = 8) and *Bos taurus* with high- ( $\geq 45$  follicles, n = 10) or low-AFC ( $\leq 13$  follicles, n = 10). Ovaries were processed for histological evaluation and the number of preantral follicles was estimated using a correction factor (Gougeon e Chainy, 1987. J Reprod Fertil, 81:433-442). Comparisons between groups were made with ANOVA ( $P \leq 0.05$ ). The mean numbers of antral follicles (mean  $\pm$  SD) in *Bos indicus* cows was  $63 \pm 8$  (G-High) and  $15 \pm 5$  follicles (G-Low), and  $59 \pm 23$  (G-High) and  $11 \pm 3$  follicles (G-Low) for *Bos taurus* females. A large variation in the number of preantral follicles was observed among animals. There was no difference between the average number of preantral follicles of *Bos indicus* of G-High ( $48,349 \pm 30,149$  follicles) or G-Low ( $33,037 \pm 31,710$  follicles), or between *Bos taurus* of G-High ( $35,050 \pm 36,060$  follicles) or G-Low ( $30,481 \pm 43,360$  follicles). It is concluded that the population of preantral follicles did not influence the population of antral follicles in *Bos indicus* and *Bos taurus* purebred cattle with high and low AFC.



A037 Folliculogenesis, Oogenesis and Superovulation

### Local blood flow changes in the preovulatory follicle of Santa Ines ewes

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**Keywords:** colour Doppler, ovulation, sheep.

An extensive vascular formation (angiogenesis) and cell differentiation (luteinization) occur in the follicular wall between LH peak and early development of corpus luteum. Recent studies indicate the colour doppler ultrasound as a non-invasive and useful tool for evaluation of ovarian vascular function, allowing real-time observation of blood flow in an enclosed area, such as preovulatory follicles wall. The aim of this study was to characterize the blood flow changes in the preovulatory follicle wall in the last phase of follicular growth before ovulation in Santa Ines ewes. Nine ewes were used in this experiment, synchronized with two PGF2 $\alpha$  doses (37.5 mg, IM; Croniben, Biogenesis), with an interval of 7 days. For the estrus observation, 48 hours after the second dose of PGF2 $\alpha$ , a marked thug was introduced to the ewes flock and every 4 hours the labeled ewes or those accepting mounts were considered in estrus (0 h). The ewes were evaluated using colour Doppler ultrasound (MyLab <sup>TM</sup> 30Gold Cardiovascular, Esaote) with a 5 MHz trans rectal multifrequency probe. The evaluation after estrus detection was performed with 4 hours intervals between assessments until preovulatory follicles ovulation. A total of 17 preovulatory follicles (greater than 4.0 mm) were evaluated. The average size of the follicle at the ovulation time was 5.85  $\pm$  0.45 mm and the average period between estrus and ovulation was 18.24  $\pm$  4.05 h. For follicular vascularization evaluation a subjective classification was used: follicles with intense vascularization were graded as 1; intermediate vascularization 2 and follicles without irrigation 3. For the comparison was used the total follicles number in each time after estrus (0, 4, 8, 12, 16 h), and classified with different degrees of irrigation (1, 2 or 3). To compare the follicles groups chi-square test (P < 0.05) was performed. Regarding follicles with moderate irrigation (2), no significant difference (P > 0.05) was observed at 0 (58%), 4 (76%), 8 (80%), 12 (50%) and 16 h (50%). At 12h there was an increase (P < 0.05) in the number of follicles with intense irrigation (50%), at 16h this pattern was maintained. Before 12 h very few follicles had intense irrigation, showing no significant differences (p > 0.05) in 0 (0%), 4 (5.8%) and 8h (13.3%). It was observed that at 8h, follicles without irrigation (6%) was significantly lower (P < 0.05) compared to that observed at 0 (41%) and 4 h (17%). At 12h there was no pre ovulatory follicle without irrigation. The results suggest that 12 hours after estrus detection occur an increase in the vascularization of preovulatory follicle wall. This difference in blood flow can be useful to determine the proximity of ovulation, and differentiate preovulatory follicles from anovulatory follicles, even when the diameter is similar between them.



A038 Folliculogenesis, Oogenesis and Superovulation

**Effect of the alterations in L-arginine/Nitric Oxide synthase/Nitric Oxide pathway on nuclear maturation and intracellular concentration of cAMP and cGMP in bovine COC's**

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**Keywords:** *in vitro* oocyte maturation, nitric oxide, nucleotides.

L-arginine (L-arg) is the precursor for the Synthesis of Nitric Oxide (NO) by the activity of the enzyme Nitric Oxide Synthase (NOS). The main pathway of NO action is through the cGMP pathway. Changes in the production of NO / cGMP have been related with the events of the resumption and progression of meiosis in oocytes. The follicular wall hemi-sections (HS) in maturation medium partially inhibit nuclear maturation of oocytes in culture. The aim of this study was to evaluate the effect of changes in L-arginine/NOS/NO pathway in the nuclear maturation of bovine oocytes and their relation with the production of intracellular nucleotides cAMP and cGMP in presence of HS. Groups of 20 COCs (140 COCs/treatment) were cultured for 22 h with 8 HS of follicular wall in incubator at 38.5°C and 5% CO<sub>2</sub> in 200 µL of maturation medium (TCM 199/BSA) supplemented with 5 mM of L-arg, 1 mM of N<sup>ω</sup>-nitro-L-arginine methylester (L-NAME) NOS inhibitor, or any supplementation (control). Oocyte nuclear maturation state was assessed by staining with 2% acetic orcein and the intracellular concentrations of cAMP and cGMP in COCs were determined at 0, 1, 3 and 6 h of culture by enzyme immunoassay technique. The results were evaluated by analysis of variance and means were compared by SNK test at 5% probability. Compared to the control, the addition of L-arg or L-NAME decreased (P<0.05) the percentage of oocytes at germinal vesicle state (VG) ( $5.9 \pm 7.6$  and  $5.9 \pm 5$  vs  $33.9 \pm 23, 8$  and increased (P<0.05) the percentage of oocytes that reached the MII state ( $27.1 \pm 6.4$  and  $72.9 \pm 14.6$  vs.  $8.4 \pm 6.5$ ) (P <0, 05). L-arg did not affect (P> 0.05) cAMP concentration in DCOs, but attenuated (P <0.05) the cGMP decrease during the first hour of culture compared with L-NAME and the control ( $0.87 \pm 0.13$  vs  $0.25 \pm 0.26, 0.33 \pm 0.20$ ). L-NAME increased (P <0.05) cAMP concentration in the first hour when compared with the control ( $0.92 \pm 0.26$  vs  $0.43 \pm 0.15$ ) and decreased (P <0.05) the concentration of cGMP at 3 h of culture in relation to the control and L-arg ( $0.07 \pm 0.50$  vs  $0.55 \pm 0.05$  and  $0.53 \pm 0.05$ ). In presence of HS of the follicular wall, changes on the L-arg/NOS/NO pathway affected the resumption and progression of oocytes meiosis. These results suggest that the events of nuclear maturation after addition of L-arg cannot be related to changes in the concentration of cAMP and cGMP. However, partial inhibition of NOS activity with the addition of L-NAME in the culture medium, change the concentration of nucleotides (cAMP and cGMP) suggesting a possible stimulatory pathway on nuclear maturation of bovine oocytes in a inhibitory *in vitro* culture system.



A039 Folliculogenesis, Oogenesis and Superovulation

### **Gene expression related to ovulatory capacity in superovulated or non-superovulated Angus cows**

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**Keywords:** bovine, MOET, ovulatory capacity.

Embryo transfer (MOET) has contributed significantly for genetic improvement of Brazilian cattle and the superestimulatory treatment P-36 has been successfully used in MOET programs. Recently, increased mRNA levels of LH receptor (LHR) and angiotensin II receptor type 2 (AGTR2) have been reported in granulosa cells of superovulated Nelore cows. The aim of this study was to evaluate the effects of the P-36 protocol on the gene expression of AGTR2 and LHR, related to ovulatory capacity, in granulosa cells of superovulated Angus cows. Multiparous Angus cows (n=17), were randomly assigned to two experimental groups: control and P-36. The control group (non-superovulated cows, n=7) received in a random day of the estrous cycle (D0) an intravaginal device containing progesterone (1.0g; Primer<sup>®</sup>; Agener Animal Health, SP, Brazil) and 2.5 mg of estradiol benzoate (EB; RIC-BE<sup>®</sup>; Agener Animal Health SP, Brazil; IM). After 8 days (D8) cows were treated with PGF2 $\alpha$  (150 mg dclprostenol; Prolise<sup>®</sup>; Agener Animal Health, SP, Brazil; IM; 07:00am) and the intravaginal device was removed (07:00pm). Animals in the P-36 group (n=10) received, at the beginning of the protocol (D0), Primer<sup>®</sup> and 2.5 mg of EB. Five days after (D5), FSH treatment was started (pFSH, 200 mg, Folltropin<sup>®</sup>; Bioniche Animal Health, Ontario, Canada, IM) and consisted of twice daily IM injections for four days, on D7, PGF2 $\alpha$  was administered and the intravaginal device was removed 36h later (D8, 07:00pm). Animals in both groups were slaughtered 12h (D9) after device removal and the ovaries were transported to the laboratory immediately after slaughter. Control dominant follicles (n=7, control group) and superovulated follicles (n=20) were dissected to obtain the granulosa cells and subsequent extraction of total mRNA. Amplification of housekeeping (cyclophilin; PPIA) and target genes was performed by real time RT-PCR according with the Sybr Green protocol. Relative gene expression values were determined by the Pfaffl method. The means of follicular diameter (mm) and the abundance of mRNA was compared by ANOVA and t test, respectively, and significance level was P<0.05. There was no significant difference in follicular diameter of the control group (13,85 $\pm$ 0,68) when compared to the P36 group (13,13 $\pm$ 0,42). The mRNA levels of LHR and AGTR2 were higher (P<0.05) in the control group (1.02 $\pm$ 0.24 and 3.76 $\pm$ 1.07, respectively) when compared to P-36 group (0.33 $\pm$ 0.04 and 1.02 $\pm$ 0.21, respectively). Therefore, our results suggest that the gene expression related to ovulatory capacity is negatively influenced by the P-36 protocol, suggesting the need for adjustments in the superestimulatory protocol when used in *Bos taurus* cattle.

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A040 Folliculogenesis, Oogenesis and Superovulation

### **Expression of genes related to oocyte competence in bovine cumulus-oocyte complexes morphologically divided in different grades**

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**Keywords:** cumulus-oocyte complex, gene expression, in vitro maturation.

Traditional methods for the evaluation of oocyte quality are based on morphological classification of the immature *cumulus*-oocyte complex (COCs) because they resemble the atresia grade of its follicle source. Although imprecise and controversial, the use of a morphological classification system is crucial for pre-selection of COCs with high development potential and able to maximize the production of more competent embryos. However, little is known about the expression of competence-related genes in morphologically different oocytes. The objective of this study was to evaluate the expression of genes related to competence in immature and IVM oocytes derived from COCs classified into grades I, II and III (n=3 replicates/group). COCs from follicles ranging 3-8 mm obtained from abattoir ovaries were separated into three groups according to the morphological classification: grade I (GI, oocytes with homogeneous cytoplasm, uniformly granular and surrounded by three layers of compact *cumulus* cells); grade II (GII, oocytes with homogeneous cytoplasm, uniformly granular and surrounded by less than three layers of *cumulus* cells) and grade III (GIII, partially denuded oocytes). Groups of 20 COCs were denuded by repeated pipetting before (group Immature) and after IVM (24 h) in TCM 199 bicarbonate supplemented with 6% BSA, pyruvate (11 µg/µL), amikacin (16.67 mg/µL), FSH (0.1 mg/mL, Pluset®, Serovet, Rome, Italy), LH (50 mg/mL, Lutropin®, Bioniche, Belleville, Ontario, Canada) and estradiol (1 µg/µL), total RNA was extracted by RNeasy® kit (Qiagen) and RNA pools of 20 oocytes were reverse transcribed by SuperScript III® enzyme (Life Technologies). Expression of growth and differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and the oocyte-secreted protein 1 (OOSP1) was investigated by real-time PCR (StepOnePlus®: Life Technologies) using PowerSybrGreen® (Life Technologies). The relative quantification method  $\Delta\Delta C_t$  was used with cyclophilin (PPIA) as the endogenous gene. The effects of the grade of the COC and the maturation period were tested by ANOVA and the groups were compared by the Tukey-Kramer HSD test. Differences were considered significant when  $P < 0.05$ . The expression of GDF9 was reduced following IVM, and inversely the OOSP1 expression was increased after IVM, while BMP15 expression did not differ. There was no effect of morphological status on the expression of the genes analyzed. Results suggest that IVM may influence the expression of genes related to oocyte competence, but the morphological discrimination did not indicate any difference in the expression of competence-related genes.

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A041 Folliculogenesis, Oogenesis and Superovulation

### **Optimization of lipofection conditions in bovine granulosa cells: preliminary results**

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**Keywords:** bovine, granulosa cells, lipofection.

Granulosa cells (GC) are important constituents of the follicular environment for oocyte competence acquisition, performing functions such as steroidogenesis, expression of LH receptors (LHR), and the synthesis of many essential proteins (Richard *et al.* 1996, Biol Reprod, 54, 22-28). Lipofection, which is used as a means to introduce interfering RNAs for gene silencing is an efficient and less aggressive transfection method, being an important tool for to investigate the role of cellular genes and proteins (Oliveira *et al.* 2,005, Genetics and Molecular Research, 4, 185-196). The aim of this work was to establish ideal conditions for lipofection in bovine GC. To obtain GC, slaughterhouse bovine ovaries had their follicles of 2-6mm aspirated and after removal of *cumulus*-oocyte complexes the cells were cultured in DMEM with 44 mM NaHCO<sub>3</sub>, 50µg/mL ciprofloxacin, 2.5 mg/mL amphotericin B and 5% FCS in 100mm petri dishes at 38.5°C and 5% CO<sub>2</sub> in air. After confluency, the GC were separated with 0.05% trypsin and 0.02% EDTA in PBS and seeded at 5 x 10<sup>4</sup> cell / well in 4-well dishes. After 48 hours of culture, the medium it was replaced with lipofection agents Lipofectamine<sup>®</sup> RNAiMAX (1, 2 and 3µL; Invitrogen, São Paulo, Brazil) or Lipofectamine<sup>™</sup> 2000 (1, 2 and 3µL; Invitrogen, São Paulo, Brazil) and the transfection indicators siGLO<sup>®</sup> (30, 50, 75 and 100 nM; Thermo Scientific Dharmacon<sup>®</sup>, São Paulo, Brazil) or FUGW transgenic plasmid prepared in the laboratory (100, 200, 300, 400, 600 and 900 nM), in 5 replicates per treatment. As control, GC groups were not exposed to drugs and cultured in conventional DMEM. The lipofection efficiency was visually verified in a subjective manner considering the proportion fluorescent GC (indicative of penetration of the lipofector with agent SiGLO<sup>®</sup> or FUGW) at 24 and 48 h of culture. The highest efficiency was observed at 24 h of culture, once at 48 h the GC were already fairly degenerate. Regarding lipofection agents, groups with Lipofectamine<sup>®</sup> RNAiMAX + siGLO<sup>®</sup> or FUGW, at all concentrations, showed a low proportion of transfected GC (20%); Lipofectamine<sup>™</sup> 2000 + FUGW groups, for all concentrations, also showed low percentage of transfected GC (30-40%), but with good fluorescence intensity. Lipofectamine<sup>™</sup> 2000 + siGLO<sup>®</sup> groups showed the highest efficiency of lipofection, both in intensity and in percentage (80-90%). Concentrations were optimized with 2µL and 100 nM, respectively. From the best conditions found at 24 h of culture, with 2µL Lipofectamine<sup>™</sup> 2000 + 100nM siGLO<sup>®</sup>, it is possible to establish a methodology for gene silencing by lipofection in GC and to use such strategy as a tool for functional analysis of genes of interest. Evaluation of the inclusion of the silencing agent (siRNA) by lipofection in the conditions defined in this experiment are underway.

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A042 Folliculogenesis, Oogenesis and Superovulation

### **Relation between antral follicle count and size of the uterus in heifers**

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**Keywords:** antral follicle, *Bos indicus*, uterus.

The relations between the number of follicles and hormone dosage were demonstrated as possible parameters to represent the reproductive potential in *Bos Taurus* females (Ireland et al., 2011, *Reprod. Fertil. Dev.* 23, 1–14). The objective of this study was to compare the relation between antral follicles population and uterine diameter in Nelore heifers. Were used during october 2012, 98 Nelore heifers with body score condition between 3,0 to 3,5 (scale 1 to 5), managed in pasture of *Brachiaria brizantha* and mineral supplementation ad libitum containing 9% phosphorus. The animals were submitted to two transrectal ultrasonography exams (7.5 mHz transducer, Mindray, China) with 10 days of interval, for evaluation of cyclicity, viewing CL in one of the two tests, counting all the visualized antral follicles (AFC) and measurements of uterine diameter, after obtaining three transversal evaluations and averaging between dimensions. The results were submitted to analysis of variance and correlation ( $P < 0,05$ ) and heifers were classified into low AFC ( $10.7 \pm 2.1$ ,  $n = 22$ ), medium AFC ( $15.0 \pm 1.0$ ,  $n = 29$ ) and high AFC ( $21 \pm 3.2$ ,  $n = 47$ ); ( $P < 0.05$ ). The uterine diameter was greater ( $P < 0.05$ ) for heifers with high AFC ( $10,7 \pm 1,6$  mm) and medium AFC ( $10.2 \pm 1.6$  mm) than the low AFC ( $9.7 \pm 1.8$  mm). Prepubertal ( $n=62$ ) and pubertal ( $n=36$ ) heifers had similar values ( $P > 0.05$ ) for AFC, resulting in  $17.1 \pm 5.1$  vs  $16.7 \pm 4.6$ , respectively. The uterine diameter was greater ( $P=0.003$ ) in pubertal ( $12 \pm 1.1$  mm) than in prepubertal heifers ( $10.2 \pm 1.8$  mm). The AFC was positively correlated with the size of the uterus ( $CR = 0.23$ ,  $p=0.01$ ). It was observed in prepubertal and pubertal heifers  $CR = 0.34$ ,  $p = 0.006$  and  $CR = 0.01$ ,  $p = 0.9$ , respectively. In conclusion, the results suggest that the AFC may influence the uterine development of prepubertal heifers.



A043 Folliculogenesis, Oogenesis and Superovulation

### **Kisspeptin stimulates LH release, is enhanced by estradiol and induces ovulation in bovine females**

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**Keywords:** Kiss1, neuroendocrinology, synchronization of ovulation.

The aim of this study was to evaluate the viability of the use of Kisspeptin (KP) in the pharmacological control of LH release and ovulation in bovine females. *EXP 1* investigated the capacity and profile of LH release of prepubertal females (absence of CL and P4<1ng/mL) of different breeds [24 Gir calves (6 - 9 m.o.) and 24 Holstein calves (4 - 8 m.o.)] after intramuscular (i.m.) administration of KP. Females were randomly distributed in two treatment-groups receiving i.m. KP or GnRH (buserelin acetate; positive control). Thirteen blood samples were collected 20 min apart to measure circulating LH. Holstein females showed higher LH released than Gir (AUC, 457.8±91.7 and 172.1±27.9 ng/mL/min, respectively; P=0.001), where KP provided similar LH response in relation to GnRH (P=0.27). KP provided maximum LH release amplitude in 20 min after treatment. *EXP 2* tested the hypothesis that LH release in response to KP stimulus is enhanced by previous E2 exposition. Eight ovariectomized Nelore females were allocated in four treatment-groups (crossover, four replicates, n=8/group): SAL (saline solution); KP (i.m. injection of KP); EB+KP [i.m. injection of KP + estradiol benzoate (EB)]; and EB+KP12 (EB injection 12 h before KP adm). The SAL group did not show LH surge. The AUC of LH was greater (P=0.02) for EB+KP12 (448.2 ± 139.2 ng/mL/min) than for KP (189.1 ± 39.1 ng/mL/min). EB+KP group presented same (P>0.05) AUC (253.6 ± 41.9 ng/mL/min) compared to KP and EB+KP12. The LH surge started very quick after KP administration, at 9.5 ± 5.8 min; 4.0 ± 6.3 min e 18.5 ± 2.7 min for KP, EB+KP and EB+KP12 groups, respectively (P>0.05). After treatments, the LH peak was observed 28.8 ± 8.8 min; 37.5 ± 20.5 min e 63.7 ± 15.8 min after treatment for KP, EB+KP and EB+KP12, respectively (P=0.22). *EXP 3* studied the KP capacity as an ovulation inducer. Holstein females (n=47) were synchronized and during the initial diestrus (seven days after AI day), females were grouped according with the presence and size of the CL and dominant follicle (DF) of the first follicular wave growth in one of the three treatments: SAL (n=12); KP (n=19); or GnRH (buserelin acetate; n=16). Blood was collected from six animals per group at -20, 0, 40, 80, 120, 160, 210, 270 min after treatments to measure circulating LH and P4. At the moment of treatment, follicles showed same size (P=0.15) as well as CL (P=0.13) and circulating P4 (P=0.16) among groups. GnRH provided higher LH release (AUC 237.60±39.43; P<0.0001) than KP (AUC = 61.43±7.16). The LH peak occurs 20 min after KP i.m. injection, which was missed in this experiment. However, KP provided the same ovulation rate (50.0%) in comparison with GnRH (37.5%; P=0.14). There was no ovulation or LH surge in SAL. Thus, i.m. application of KP was effective to induce a LH pre-ovulatory surge; it was enhanced by previous exposition of E2 and induced ovulation in bovine females.



A044 Folliculogenesis, Oogenesis and Superovulation

### Comparison of follicular irrigation of mares supplemented and non-supplemented with L-arginine - partial results

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**Keywords:** doppler ultrasonography, L-arginine, mare.

The vascularization of the preovulatory follicle has been positively correlated with pregnancy rates in mares<sup>1</sup>. In a previous study<sup>2</sup>, the intake of the amino acid L-Arginine (Arg) caused an increase in uterine blood flow and resulted in acceleration of postpartum uterine involution in mares. Therefore, the present study aimed to evaluate vascular perfusion of ovaries and follicles at the time of induction of ovulation in mares supplemented or not with Arg. Sixteen mares between 3 and 8 years old were randomly divided in two groups: mares supplemented with Arg (n = 8) and mares non-supplemented with Arg (n = 8). The animals received 100 g Arg (Ajinomoto Amino Science LLC, Raleigh, NC, USA) with 3 kg of feed from the eighth day after ovulation in portions twice a day. B mode ultrasound examination was performed to assess the follicular dynamics. At the time when a follicle  $\geq 35$  mm in diameter was detected, the ovarian and follicular vascular perfusion was assessed by spectral and color Doppler ultrasonography mode. In color Doppler mode, follicles vascularization were subjectively estimated and graded in percentages (0 to 100) of the follicular wall with signs of vascularization. In spectral Doppler mode, the ovarian artery ipsilateral to the preovulatory follicle was examined and the ovarian vascular perfusion evaluated by the resistance (RI) and pulsatility index (PI). To obtain these indexes, the cursor was positioned in an artery of the ovarian pedicle and three identical spectral graphics of subsequent cardiac cycles were generated to obtain the RI and PI values. After the evaluations, ovulation was induced with 1 mg of deslorelin acetate and detected between 24 and 48 hours after induction. Statistical analysis was performed with SAS (Release 9.2, SAS Institute, Inc., Cary, NC, USA). The distribution of variable responses was analyzed by the Shapiro-Wilk test, and differences between groups were evaluated by the LSD test (Least Significant Different test) and non-paired t-test. The level of statistical significance was set as 0.05. No statistical differences were found for the variables PI (P = 0.42), RI (P = 0.60) and subjective follicular vascularization (P = 0.65) between Arg supplemented and non-supplemented mares. The indexes represented inverse relationship with vascular perfusion of the target tissue. In young mares, Arg was not efficient to increase vascular perfusion of the preovulatory follicle.

#### References:

<sup>1</sup>Silva LA, Gastal EL, Gastal MO, et al: Relationship between vascularity of the preovulatory follicle and establishment pregnancy in mares. *Anim. Reprod.* 2006; 3:339-346; <sup>2</sup>Mortensen CJ, Kelley DE, Warren LK. Supplemental L-Arginine shortens gestation length and increases mare uterine blood flow before and after parturition. *J. Eq. Vet. Sci.* 2011; 1-7.



A045 Folliculogenesis, Oogenesis and Superovulation

### Ovarian antral follicular population in cows of Nelore and Girolando breeds

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**Keywords:** antral follicles, Girolando, Nelore.

The ovarian antral follicular population (OAFP) is positively associated with fertility in cattle (Ireland et al., 2008, *Biology of Reproduction*, 79, 1219–1225). In previous studies, dairy cows with low OAFP had lower reproductive performance when compared to cows with higher OAFP (Mossa et al., 2012, *J. Dairy Sci.*, 95, 2355–2361). The growing interest in dairy breeds adapted to the tropics demands more studies on this topic. This study aimed to evaluate the OAFP in Nelore (n = 13; *Bos taurus indicus*) and Girolando cows (n = 19; *Bos taurus indicus* ¼ x *Bos taurus taurus* ¾). All animals received an intravaginal progesterone device (CIDR®, InterAg, Hamilton, New Zealand), 2 mg IM of estradiol benzoate (Estrogin®, Biofarm, Jaboticabal, São Paulo, Brazil) and 25 mg IM of Dinoprost (Lutalyse®, Pfizer, Guarulhos, São Paulo, Brazil) on a random day (D0) to synchronize the emergence of a new follicular wave. After seven days (D7) the devices were removed and on day 11 (D11) antral follicles were counted using an ultrasound machine equipped with a 7.5 MHz transducer (Aquila®, Pie Medical, Maastricht, The Netherlands). All follicles ≥ 3 mm in diameter were counted in both ovaries. The counting procedure was repeated three times with intervals of 35 days between consecutive measurements. To evaluate the effect of breed on OAFP counts, ANOVA followed by Tukey test was used considering P<0.05 for significant differences (ASISTAT 7 6-beta; 2013). No significant difference was observed between OAFP in Nelore and Girolando females. In Nelore, the average OAFP was 31.48 ± 9.83, with no significant variation between the three counts (35.15, 33.61 and 25.69). In Girolando females, the average OAFP was 27.01 ± 11.25, and similarly there was no significant variation between the OAFP counts (30.26, 27.47 and 23.31). It was concluded that OAFP of adult Nelore and Girolando females is similar and remains constant during consecutive estrous cycles when animals are maintained under the same experimental conditions.



A046 Folliculogenesis, Oogenesis and Superovulation

### **Establishment of a model for the study of follicle dominance in cows based on follicle aspiration in vivo**

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**Keywords:** dominance, follicle deviation, in vivo aspiration.

One of the main challenges for the study of follicular dominance in cattle is to determine the exact moment that follicle deviation occurs. Studies show that European breeds have follicular deviation at around 8.5 to 9.0 mm, whereas in Nelore females, deviation occurs between 5.5 and 7.0 mm (reviewed by Sartori et al., Repr. Dom. Anim., v.7, p.357-375, 2010). However, an individual variation does not allow us to precisely define when deviation will occur. The objective was to establish a model for the study of follicular dominance, identifying more precisely the exact time of occurrence of follicular dominance. Thus, 11 Nelore (NEL) and 10 Holstein cows (HOL) were used in a crossover design. All cows had the emergence of a new wave synchronized by OPU of follicles > 7 mm. Cows were then implanted with norgestomet and 24 h later, two injections of PGF2 $\alpha$  were given 12 h apart. CL regression was monitored by ultrasound. Ovarian ultrasound evaluation was also performed every 12 h for follicle measurements. The biggest growing follicle (F1) reached the expected deviation for each breed (NEL = 6.5 mm, HOL = 8.5 mm) at 72  $\pm$  12 h after the onset of the study. At this time, cows were randomly allocated into two groups (C: control, T: Treatment). The cows in group T had the F1 aspirated, and in group C there was no aspiration. Ultrasound evaluation of the control and the other follicles (F2 and F3) were performed until 72 h after deviation. The data were analyzed by generalized linear models (SAS<sup>®</sup> 9.2) and presented as least squares means  $\pm$  SE. In group C, 12 h after F1 reached its expected size at deviation, it was bigger than the others (F2 and F3), featuring dominance. No difference was observed between the diameter of F1 at the time of aspiration and F2 12 h later in the T group for both NEL (F1: 6.9 $\pm$ 0.20 vs F2: 7.0 $\pm$ 0.15 mm; P=0.0003) and HOL (F1: 8.8 $\pm$ 0.35 vs F2: 8.5 $\pm$ 0.29 mm; P=0.0003). Likewise, there was no difference in diameter between F1 of group C and F2 of group T after aspiration of F1 of group T over the time of evaluation. However, F2 of group T had a bigger diameter (P<0.05) as compared to F2 of group C from 24 h after the time of deviation for NEL and HOL. It is concluded that the second largest follicle is able to become morphologically dominant when the largest follicle is aspirated, allowing for a more precise estimation of the exact moment of dominance, established by another follicle.

**Acknowledgments:** FAPESP and CNPq.



A047 Folliculogenesis, Oogenesis and Superovulation

### **Hormonal evaluation during the ovulatory cycle of Holstein, Gir and Buffalo heifers at the same environment and nutritional management**

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**Keywords:** heifers, hormones, interovulatory interval.

The objective of the present study was to evaluate during the same interovulatory interval, plasma concentrations of P4, testosterone, insulin and IGF-I in Holstein (n=14), Gir (n=5) and Buffalo (Murrah cross; n=15) heifers, maintained under the same nutritional management. All heifers were kept at the Department of Animal Reproduction (CBRA/FMVZ/USP), Pirassununga Campus. They were pre-synchronized with two doses of prostaglandin F2 $\alpha$  (D-cloprostenol, 150 $\mu$ g, IM; Prolise<sup>®</sup> Tecnopec, São Paulo, SP), fourteen days apart (D0 = day of first ovulation). From D0, daily ultrasound scan of the ovaries and blood collection from their jugular vein were performed until the detection of the following ovulation. The CL volume was calculated based on the equation for volume of a sphere  $(3/4) * (\pi) * (\text{radius})^3$ . The CL cavity volume was disregarded when present. P4 analysis (RIA kit, Coat-A-Count, Siemens Medical Solution Diagnostic<sup>®</sup>, Los Angeles, CA) was performed every day during the interval whereas testosterone analysis (RIA kit, Coat-A-Count, Siemens Medical Solution Diagnostic<sup>®</sup>, Los Angeles, CA) was performed from D0 to D13 of the interval. Insulin (RIA kit, Coat-A-Count, Siemens Medical Solution Diagnostic<sup>®</sup>, Los Angeles, CA) and total IGF-I (Elisa kit Quantikine<sup>®</sup> ELISA, Human IGF-I, R&D Systems<sup>®</sup>, Minneapolis, MN) were measured in two phases: the follicular phase (D0 and D1) and the luteal phase (D9 to D13). The response variables were analyzed by PROC GLIMMIX for repeated measures of SAS software (SAS System for Windows, 9.3; SAS Institute Inc., Cary, NC). P4 concentrations were affected by day (P<0.0001) and by interaction breed\*day (P=0.001). These effects were observed for testosterone concentrations [breed, (P<0.0001); day (P<0.0001), interaction (P=0.001)]. The results showed that Holstein heifers presented similar concentrations of P4 and lower concentrations of testosterone than Gir heifers. The bubaline heifers presented the lowest concentration of both steroids. Regarding CL volume, it was observed effect of breed (P<0.0001), day (P<0.0001) and their interaction (P<0.0001). CL volume was greater in taurine heifers. Buffalo heifers showed the smallest CL volume during the entire interval. IGF-I (P<0.0001) and insulin (P<0.0001) concentrations were higher in the follicular (129.5 $\pm$ 4.5ng/ml and 7.1 $\pm$ 0.7UI/ml, respectively) than luteal phase (102.8 $\pm$ 4.5ng/ml and 4.7 $\pm$ 0.7UI/ml, respectively) for the three breeds. Moreover, insulin concentration was affected by breed (P=0.025). Gir heifers (9 $\pm$ 2.1UI/ml) presented higher levels of insulin when compared to the Holstein heifers (4.1 $\pm$ 0.6UI/ml). However, IGF-I levels did not differ among breeds (P=0.054). Results presented herein may indicate that breed differences in steroid production or liver metabolism could have an effect on circulating P4 and testosterone concentrations.



A048 Folliculogenesis, Oogenesis and Superovulation

### **A role for exosomes in regulation of TGF- $\beta$ family members during equine ovarian follicular development**

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**Keywords:** exosomes, follicular fluid, miRNAs.

During ovarian follicular development, cell communication is a crucial and well-regulated event, culminating with follicular ovulation or atresia. These events are dependent on endocrine, paracrine and autocrine signaling. TGF $\beta$  signaling is key in follicular development and consequently ovulation and oocyte competence (Knight, 2006, *Reproduction*, 132, 191-206). Exosomes are cell-secreted vesicles between 40-100 nm in size, and contain bioactive materials such as miRNAs and proteins (Raposo, 2013, *Journal of Cell Biology*, 200, 4, 373-383). Exosomes can be taken up by target cells through different endocytotic pathways and mediate the delivery of mRNA, protein and miRNAs. Recently, we described the presence of exosomes in follicular fluid that can be taken up by granulosa cells (da Silveira, 2012, *Biology of Reproduction* 86, 71). Our hypothesis is that regulation of TGF $\beta$  signaling members in granulosa cells during follicle development is mediated by exosomes secreted in ovarian follicular fluid. In order to test this hypothesis, granulosa cells and follicular fluid were collected from ovarian follicles (35mm size; immature, n=4) and 34h after GnRH/LH stimulation (mature, n=4). Real-time PCR was used to investigate 18 members of the TGF $\beta$  family in freshly isolated granulosa cells before culture and granulosa cells in culture exposed for 24h to exosomes (EXO), student's t-test was used to compare the treatments. *ACVR1* (P<0.05) and *ACVR2B* (P<0.05) levels were decreased in granulosa cells following EXO treatments compared to no treatment. For SMAD target genes, *CDKN2B* (P<0.03) levels in granulosa cells were increased following EXO treatments, while both *ID1* (P<0.02) and *ID2* levels were decreased (P<0.02) in granulosa cells by treatment with EXO from immature follicles. Therefore, treatment with exosomes originating from immature follicles leads to altered gene expression of selected TGF $\beta$  family members in granulosa cells from mature follicles. Interestingly, we identified high levels of *ACVR1* and miR-27b (a predicted regulator of *ID2*) in exosomes isolated from mid-estrous follicles. We are currently investigating the presence of miRNAs in EXO isolations in order to identify miRNAs involved in regulating TGF $\beta$  family members.



A049 Folliculogenesis, Oogenesis and Superovulation

### Differences in lipid profiles of oocytes recovered by ovum pick-up from *Bos indicus* and 1/2 *indicus-taurus* with high versus low oocyte yield

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**Keywords:** antral follicle count, lipid content, oocyte yield.

The objective of this study was to compare the overall lipid profile of oocytes recovered from *Bos indicus* (Nelore) and *indicus-taurus* (1/2 Nelore x Angus) cows with high versus low oocyte yield and to correlate such profiles with the mechanisms involved in oocyte competence. Nelore (n = 20) and 1/2 Nelore X Angus (n = 20) cows were randomly subjected to follicular aspiration during their estrous cycle (D0) to withdraw all follicles  $\geq 3$  mm and to induce the growth of a new follicular wave. Ovaries were examined by ultrasonography during days 4, 19, 34, 49 and 64, and antral follicles  $\geq 3$  mm were counted. The last ultrasound (D64) evaluation was performed after the animals were slaughtered. Cows were then assigned to one of two groups: a) high antral follicle count (AFC) / oocyte yield (mean  $\geq 30$  follicles; Nelore NH-group, n = 3; 1/2 Nelore x Angus, AH-group, n = 5) and b) low AFC ( $\leq 15$  antral follicles; Nelore, NL-group, n = 4; 1/2 Nelore x Angus, AL-group, n = 4). A new OPU procedure was performed on D49 to obtain the oocytes used in this study. Following OPU recovery, the lipid profiles of the oocytes were obtained via matrix-assisted desorption/ionization – mass spectrometry (MALDI-MS). Profiles constituted of ions corresponding to sphingomyelin (SM), phosphatidylcholine (PC) and triacylglycerol (TAG) were observed. Data was submitted to partial least squares-discriminant analysis (PLS-DA). COCs from preantral follicles and expanded oocytes presented differences in the abundance of membrane structural lipids compared to GI, GII and GIII quality oocytes. Considering just GI, GII and GIII oocytes, Nelore samples tend to cluster separately from 1/2 Nelore x Angus samples, specially the NH-group. There were no differences among oocytes from 1/2 Nelore x Angus (AH- and AL-groups). The lipid ions [PC (P-38:5) + H]<sup>+</sup> and/or [PC (P-36:2) + Na]<sup>+</sup>, [PC (38:2) + H]<sup>+</sup>, [PC (38:5) + Na]<sup>+</sup> and [TAG (60:8) + NH<sup>4</sup>]<sup>+</sup> were more abundant in the Nelore oocytes (NH- and NL-group) compared to 1/2 Nelore x Angus (AH- and AL-groups). There are therefore membrane structural differences, and also in the lipid droplets, between Nelore and 1/2 Nelore x Angus oocytes recovered from cattle with high versus low oocyte yield. The greater abundance of some PC in Nelore oocytes may be related to lipidic metabolic rate differences and contribute to oocyte competence and embryo development.



A050 Folliculogenesis, Oogenesis and Superovulation

### **Temporal expression of BMPRII and EGFR genes in bovine cumulus cells and oocytes during maturation *in vitro*-preliminary results**

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**Keywords:** BMPRII, cumulus cells, EGFR.

GDF-9 and BMP15 are important oocyte-derived paracrine factors that control cumulus cells function (Gilchrist et al. 2008, Human Reproduction Update, 14, 159–177). These factors use a combination of signaling systems including the TGF $\beta$  superfamily, BMPRII receptor. EGFR is essential for LH signaling in COCs to induce maturation and assist paracrine factors GDF-9 and BMP15 in cumulus cells expansion (Su et al. 2010, Molecular Endocrinology, 24, 1230-1239). Gene silencing by lipofection in cumulus cells may be used to study the interaction between cumulus cells and oocyte and to evaluate the functions of cumulus genes on oocyte maturation and competence. The aim of this study was to evaluate EGFR and BMPRII expression to determine their temporal expression pattern during *in vitro* maturation in bovine oocytes and cumulus cells and determine whether they could be candidate-genes for gene silencing studies. For this purpose, genes must have stable or increased expression during culture. Cumulus-oocyte complexes were aspirated from abattoir ovaries, transferred in groups of 23 to 90 $\mu$ L drops of TCM 199 supplemented with FSH (0.5 $\mu$ g/ml), LH (5 $\mu$ g/ml), cysteine (100mM), sodium pyruvate (0,25mM), 10% FCS and gentamicin (25  $\mu$ g/ml) and cultured for 24 hours at 38.5°C and 5% CO<sub>2</sub> in air. For transcripts detection, every 3 hours, cumulus cells and oocytes samples were separated by pipetting COCs (pools of 23) and then frozen in PBS solution containing 0.1% polyvinyl alcohol and 100 IU/mL RNaseOUT inhibitor (Invitrogen™ - S.P./Br.), plunged into liquid N<sub>2</sub>, and stored at -80°C for later analysis. RNA isolation was performed with TRIzol reagent (Invitrogen™- S.P./Br.) and reverse transcription with the enzyme MultiScribe™ Reverse Transcriptase (Applied Biosystems® - S.P./Br.), following the manufacturers recommendations. Relative quantification of gene transcripts was performed by real-time PCR using SYBR green PCR kit master mix (Applied Biosystems® - Brazil) with 3 endogenous controls (PPIA, GAPDH and  $\beta$  actin). Time effects were tested by ANOVA and means were compared by Student's t test (3 replicates). In oocytes, EGFR and BMPRII expression was stable throughout maturation (P>0.05). In cumulus cells, EGFR expression was increased at 3, 6, 9 hours compared to other periods (P<0.05). BMPRII expression increased after 12 hours and decreased after 18 hours. At 6 hours, there was an inverse pattern between EGFR and BMPRII expression, as EGFR decreased BMPRII expression increased at this time point. In conclusion, this study suggests that both selected genes may be suitable candidates for gene silencing studies in oocytes. In cumulus cells, they may be candidates as long as silenced for up to 6 h for EGFR and after 6 h for BMPRII. Also, the results indicate a temporal relationship between genes, with sequential expression of EGFR followed by BMPRII, which must be investigated and compared with previous studies like Caixeta et al. 2012.



A051 Folliculogenesis, Oogenesis and Superovulation

### Effects of supplementation with detoxified castor meal on gene expression in goat oocytes

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**Keywords:** byoproducts of biodiesel, goats, oocyte maturation.

Currently, biodiesel production from oilseed plants (*Ricinus communis*) in the northeast of Brazil receives great incentives, mainly in the semi-arid region. However, this process results in great environmental concerns regarding the residues, due to the presence of ricin, a potentially toxic compound. Despite the presence of this compound in castor meal, previous studies demonstrated this product as having a high protein content (41%), making it attractive to be used as an alternative dietary source for ruminants (Diniz et al., 2011, *Livestock Science*, 135, 153-167). To evaluate the effect of feeding detoxified castor meal on oocyte viability and quality, the expression of BMP15 and GDF9 genes were measured in oocytes from 25 mixed breed adult goats allocated into two balanced dietary groups: diet I: Tifton hay and concentrate (n=12); and diet II: Tifton hay and concentrate with 15% detoxified castor meal substituting soybean meal (n=13). After sixteen months of feeding, estrus was synchronized and all animals were subsequently slaughtered. Following ovarian recovery, only grade I, II and III *cumulus*-oocyte complexes (COCs) were selected for IVM for 22 h, being subsequently analyzed by multiplex qPCR. The expression of GDF9 and BMP15 genes was evaluated using pools (4 to 5 oocytes/pool) of denuded oocytes at grades I (n = 3), II and III (n = 4) with and without the presence of the first polar body. RNA extraction, reverse transcription and qPCR were done with the *CellsDirect™ One-Step qRT-PCR* kit (Invitrogen, Paisley, UK), using 400 μM of each primer and 80 μM of a specific TaqMan® probe (Invitrogen, Paisley, UK). Results were normalized using the  $2^{-\Delta\Delta C_t}$  method with ribosomal protein S9 (RPS9) as the housekeeping gene. Relative mean values were compared by the Fisher test (P<0.05). No dietary effect was observed on BMP15 and GDF9 mRNA expression (BMP15 – 1.925 ± 0.218 diet I e 2.474 ± 0.248 diet II; GDF9 – 4.602 ± 0.779 diet I and 3.604 ± 0.557 diet II) between goat oocyte categories. However, irrespective of oocyte grade and diet, expression of BMP15 was higher in oocytes without polar body (3.466 ± 0.452 without polar body and 1.190 ± 0.087 with polar body; P<0.05); and expression of GDF9 was higher in matured oocytes (6.198 ± 0.934 with polar body and 1.489 ± 0.270 without polar body; P<0.05). In conclusion, expression of GDF9 and BMP15 genes was not affected by diet, suggesting that feeding detoxified castor meal did not change oocyte quality in goats.



A052 Folliculogenesis, Oogenesis and Superovulation

### Evaluation of the luteal function in sheep using color doppler ultrasonography

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**Keywords:** color Doppler, luteal dynamic, sheep.

The aim of the present study was to evaluate the use of color Doppler ultrasonography in the study of luteal function in sheep. Estrus was synchronized in nulliparous Santa Ines ewes (n=18) with short-term protocol of 6 days (Cavalcanti, R. Bras. Zootec., v. 41, p.1412-1418, 2012). After estrus detection, ovulation (D0) and subsequent corpus luteum (CL) development was assessed every 24 hours by ultrasonographic exams using a portable ultrasound device with color Doppler (Sonoscape<sup>®</sup> S6, Shenzhen, China), equipped with a 7.5 MHz linear transducer. Ultrasonography exams were performed until detection of a subsequent ovulation. In each sonogram, the ovaries were scanned with both B and color flow Doppler modes and a video was recorded. Luteal tissue and vascularization areas were also measured in a single 2D image (at CL largest diameter). Blood samples were collected daily for plasma progesterone (P4) determination by radioimmunoassay. Results were evaluated by ANOVA and differences between means were determined by Tukey's test. Correlations were analyzed using Pearson's correlation method. Results are shown as means±SD. Color Doppler ultrasonography allowed an early examination of the developing CL, which was first visualized at day 0.77±0.62 with an average area of 29.68±13.21 mm<sup>2</sup>. As expected, luteal dynamics was characterized by a luteogenesis period, in which a progressive increase of the luteal tissue area and plasma P4 concentration was observed (maximum 124.0±38.0 mm<sup>2</sup> and 11.23±4.89 ng/mL, respectively), a plateau phase in which no significant increase was detected, and a luteolysis period in which plasma P4 concentration decreased abruptly while luteal tissue area decreased gradually. Similarly, vascularization area progressively increased during luteogenesis (maximum 52.78±24.08 mm<sup>2</sup>) and gradually decreased during luteolysis. These results demonstrate the importance of a functional vascular structure for CL development. Also, variations observed with color Doppler ultrasonography were directly related to luteal function. A positive correlation between CL vascularization area and plasma P4 concentration during luteogenesis (r=0.22, P<0.05) and luteolysis (r=0.48, P<0.05) was observed. The low correlation value observed during luteogenesis period was likely due to high vascularization of the early CL (corpus hemorrhagicum). When CL was first visualized, mean vascularization area was 12.26±6.9 mm<sup>2</sup>, which represented 44.3% of the luteal tissue area. In conclusion, luteal dynamics in Santa Ines sheep was similar to the patterns observed in other sheep breeds and domestic ruminant species (cows and goats). Color Doppler ultrasonography allowed an early visualization of the CL, but its use to quantitatively assess luteal function remains a challenge.



A053 Folliculogenesis, Oogenesis and Superovulation

### **Effects of epidermal growth factor (EGF) on the morphology and activation of ovine primordial follicles *in situ***

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**Keywords:** ewe, oocyte, ovary.

Epidermal Growth Factor (EGF) promotes the transition of primordial to primary follicles in goats (Celestino et al., 2009, *Reprod. Sci.*, v.16, p.239-246). However, few studies were performed in sheep. The aim of this study was to evaluate the effects of EGF on the morphology and *in vitro* development of ovine preantral follicles. After collection of ovine ovaries (n=10) in the slaughterhouse, the cortex was divided and one fragment was used for histology (fresh control), while the remaining fragments were *in vitro* cultured for 7 days in supplemented  $\alpha$ -Minimal Essential Medium ( $\alpha$ -MEM- GIBCO-Invitrogen, St Louis, EUA), in the absence (control medium) or presence of EGF (1, 10, 50, 100 or 200 ng/mL). After culture, morphological analysis of preantral follicles was performed, and the follicles were classified as normal or atretic, as well as primordial or developing follicles (intermediate, primary and secondary follicles). The percentage of normal, primordial and developing follicles were compared by ANOVA and Tukey test ( $P<0.05$ ). After 7 days of culture, all treatments significantly reduced the percentage of morphologically normal follicles compared with the fresh control, where follicles had a centralized oocyte without pyknotic bodies and cytoplasmic retraction, and layers of granulosa cells well organized. However, no differences ( $P>0.05$ ) were observed between MEM and different concentrations of EGF regarding this parameter. In comparison with the fresh control, a significant decrease of primordial follicles and an increase of developing follicles were observed in all treatments, except in cultures with 200 ng/ml of EGF. In addition,  $\alpha$ -MEM and 1 ng/ml of EGF promoted higher ( $P<0.05$ ) follicular activation than the other treatments. In conclusion, the  $\alpha$ -MEM culture medium alone or supplemented with 1 ng/ml of EGF promotes ovine primordial follicles activation *in vitro*.



A054 Folliculogenesis, Oogenesis and Superovulation

### **Estimate of preantral follicles population in prepubertal and adult bitches**

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**Keywords:** canine, preantral follicles, prepubertal.

Information on the distribution and quantification of preantral ovarian follicles in bitches are scarce in the literature, especially in prepubertal bitches. The comparison between prepubertal and adult bitches will contribute for the use of reproductive biotechnologies. The objectives of this study were to estimate the population of preantral follicles in small and medium-sized bitches at prepubertal and adult ages, and to compare the population between the right and left ovaries. Eighty ovaries obtained by elective ovariohysterectomy from 40 bitches were divided into 4 groups (prepubertal small size, n = 10; prepubertal medium size, n=10; adult small size, n = 10; adult medium size, n=10). After surgery, ovaries were immersed in Bouin's fixative for histological processing. Subsequently, the ovaries were dehydrated in alcohol, fixed in xylene, embedded in paraffin and serially sectioned every 5  $\mu$ m. Every 70 histological sections were made with a blade tissue and stained with periodic acid-Schiff (PAS) and hematoxylin. The number of preantral follicles was estimated by counting the follicles in each section using the oocyte nucleus as a marker and a correction factor (Gougen and Chainy, 1987). Preantral follicles were classified according to the developmental stage. The Kruskal-Wallis test followed by the Dunn's test was used to compare groups, and  $P \leq 0.05$  was considered significant. Prepubertal medium bitches ( $\pm 12.65$  kg) had more preantral follicles compared to the other groups. The population of preantral follicles was variable between individuals of the same age and between groups (mean  $\pm$  standard error;  $51611 \pm 18577$  for small prepubertal females,  $143562 \pm 21718$  for medium prepubertal bitches,  $49546 \pm 10951$  for small adult bitches, and  $28090 \pm 15705$  for medium adult bitches). The estimates of the population of preantral follicles were different for the right and left ovaries. In conclusion, the use of the ovaries of prepubertal bitches will provide a greater number of preantral follicles for assisted reproduction techniques and the use of only one of the ovaries to estimate the population of preantral follicles in bitches may overestimate or underestimate the results.



A055 Folliculogenesis, Oogenesis and Superovulation

### Effects of kit ligand (KL) on Cyclin B1 and Y box Binding Protein 2 mRNA expression in bovine oocytes submitted to *in vitro* maturation

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**Keywords:** IVM, Kit ligand, oocyte.

The oocyte and surrounding cumulus cells (DC) secrete paracrine factors that regulate cumulus-oocyte complex (COC) differentiation. We have recently reported that oocyte-derived bone morphogenetic protein 15 (BMP15) and fibroblast growth factor 10 (FGF10) cooperate to increase kit ligand (KL) mRNA expression in cumulus cells, which stimulates meiosis progression during *in vitro* maturation (IVM) in cattle (Lima *et al.* in SSR 2012 Annual Meeting, abstract 295). Cyclin B1, a subunit of maturation promoting factor (MPF; Liu *et al.*, 2012 Theriogenology 78, 1171-1181), and Y box binding protein 2 (YBX2), a regulator of mRNA stability and spindle formation (Medvedev *et al.* Biology of Reproduction 85, 575-583), are expressed by the oocyte and are required for meiosis resumption. The aims of this study were to assess the expression patterns of Cyclin B1 and YBX2 mRNA during IVM (Experiment 1), and to test the effects of KL on cyclin B1 and YBX2 mRNA expression in bovine oocytes submitted to IVM (Experiment 2). Groups of 20 immature COCs (grades 1 and 2) from 3-8mm follicles of abattoir ovaries were used throughout the study. In Exp. 1, COCs were cultured for 1, 4, 8, 12, 16 and 22 hours (n = 4 per time point; immature COCs were used to represent 0h) in 100µl drops of maturation medium (TCM 199 containing Earle's salts supplemented with 0.4% BSA; 1µg/mL FSH; 10UI/mL LH; 22µg/mL sodium pyruvate and 75µg/mL ampicillin). In Exp. 2, COCs were cultured for 22 hours as described above with graded doses of KL (0, 10, 50 and 100ng/ml, n=4 per dose). In Exps. 1 and 2, cumulus cells and oocytes were separated mechanically after culture, total RNA was extracted from oocytes using RNeasy (Qiagen), and 100ng of RNA was treated with DNase and submitted to reverse transcription using OligoDT and Sensiscript (Qiagen). Messenger RNA abundance of cyclin B1 and YBX2 was assessed by real time RT-PCR (normalized by cyclophilin) in oocytes from Exps. 1 and 2. Treatment effects were tested by ANOVA and means were compared by the Tukey-Kramer HSD test. In Exp. 1, cyclin B1 mRNA abundance did not change during the first 12h of culture in the oocyte, but increased from 12 to 22h of culture. In contrast, oocyte YBX2 mRNA levels decreased gradually during IVM and were four times lower at 22h compared with 0h. In Exp. 2, KL did not affect cyclin B1 mRNA expression, but caused a three fold increase in oocyte YBX2 mRNA abundance at all doses tested (P=0.02). In conclusion, the present data suggest that the mechanisms by which KL stimulates meiosis progression during IVM in cattle appear to include up-regulation of YBX2 expression in the oocyte.

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A056 Folliculogenesis, Oogenesis and Superovulation

### Expression of genes associated with steroidogenic and ovulatory capacity in Angus cows undergoing fixed-time artificial insemination protocol

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**Keywords:** gene expression, ovulation, TAI.

In fixed-time artificial insemination (FTAI) protocols the diameter of the preovulatory follicle is modulated also by progesterone (P4) exposure length. Exposure to P4 may affect the future ovulatory follicle, which reflects in the quality of the oocyte and the subsequent embryo. Thus, the objective was to study the effects of P4 on the expression of genes related to ovulatory and steroidogenic capacity (LHR, AGTR2, FSHR e CYP19A1) in granulosa cells derived from Angus cows submitted to FTAI protocol. Multiparous Angus cows (n=14) were randomly assigned to two experimental groups: P4/12h and P4/24h. The animals from group P4/12h (cows slaughtered 12 h after implant removal P4; n = 7) received in a random day of the estrous cycle (D0) an intravaginal device containing progesterone (1.0 g; Primer<sup>®</sup>; Agener Animal Health, SP, Brazil) and 2.5 mg of estradiol benzoate (EB; RIC-BE<sup>®</sup>; Agener Animal Health SP, Brazil; IM). After 8 days (D8) cows were treated with PGF<sub>2</sub> $\alpha$  (150 mg d-cloprostenol; Prolise<sup>®</sup>; Agener Animal Health, SP, Brazil; IM) and Primer<sup>®</sup> was removed (19:00pm). The animals from P4/24h (cows slaughtered 24 h after implant removal P4, n = 7) were treated with a protocol similar to that described above, except that the P4 was withdrawn 12 h before (D8; 07h00min). All animals were slaughtered at D9 (07h00min) and the ovaries were transported to the laboratory immediately. Only the dominant follicle from each animal was dissected to obtain granulosa cells, which were used for extraction of total mRNA. Expression of target genes and of the constitutive gene cyclophilin A (PPIA) was assessed by real time RT-PCR with the Sybr Green protocol. Relative gene expression values were determined by the Pfaffl method. Messenger RNA levels (mean  $\pm$  SEM) were compared by the t test and the significance level was P<0.05. There was no significant difference between mRNA levels of AGTR2, FSHR e CYP19A1 in granulosa cells from groups P4/12 (3.76 $\pm$ 1.07, 0.91 $\pm$ 0.16 and 0.96 $\pm$ 0.13, respectively) and P4/24h (3.84 $\pm$ 1.42, 1.06 $\pm$ 0.05 and 1.58 $\pm$ 0.31, respectively). However, mRNA expression of LHR was higher in granulosa cells in group P4/12h (1.02 $\pm$ 0.24, P<0.05) compared to P4/24h (0.40 $\pm$ 0.19). Thus, it is suggested that the exposure length to the P4 intravaginal device in FTAI protocols might affect the expression of genes related to steroidogenic and ovulatory capacity in follicles from *Bos taurus* cattle.

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A057 Folliculogenesis, Oogenesis and Superovulation

### Luteinizing hormone receptor gene expression during follicular divergence in *B. taurus* vs *B. indicus* dairy breeds

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**Keywords:** follicular dominance, follicular dynamic, steroidogenesis.

The establishment of follicular dominance is a key phenomenon to determine the specie-specific ovulation rate, and involves the transition from FSH to LH-dependence by the dominant follicle. This physiological event occurs at different moments in *B. taurus* and *B. indicus* cattle. Previous studies of our group analyzed the relationship between the diameter at deviation and the progression of the intrafollicular steroidogenesis in dairy breeds (Arashiro et al., *Reprod Fertil Dev* 25:235, 2013). The objective of the current study was to evaluate the expression of the LH receptor (LHR) gene during follicular deviation in two dairy breeds. Mural granulosa cells (GC) were recovered from Holstein (*B. taurus*; n=10) and Gir (*B. indicus*; n=10) heifers, as described before (Arashiro et al., *Reprod Fertil Dev* 24:175, 2012). GC were collected by ultrasound-guided follicular aspiration of follicles at 6, 8, 10, and 12 mm in diameter from Holstein heifers, and 4, 6, 8 and 10 mm from Gir heifers. The recovered follicular fluid was centrifuged and the cells were washed with NaCl 0.9% saline and kept in RNA Later (Ambion, Austin, TX, USA). Total RNA extraction was performed using the RNeasy Micro Kit (Qiagen, Hilden, Germany), quantified in spectrophotometer (Nanodrop), and cDNA was synthesized using the Superscript III kit (Invitrogen, Carlsbad, CA, USA). The obtained cDNA underwent real-time PCR, using LHR specific primers in a region without occurrence of isoforms and thus producing a single fragment, a primer pair for the CYP17A1 gene as a marker of thecal cell contamination, and a primer pair for the GAPDH gene as an endogenous control. Samples with thecal cell contamination were discarded. Results were analyzed by the software REST<sup>®</sup> and are presented as means±SEM. In both breeds, LHR expression was identified in follicles of all size categories. The expression of LHR in 4 and 6 mm follicles (for Gir and Holstein, respectively) was used as a reference value (=1). A peak in LHR expression (11.0±5.8 and 10.7±8.0 -fold the reference value) was observed in 10 mm (Holstein) and 8 mm (Gir) follicles, i.e., diameters only reached after deviation in these breeds (8.6±0.4 and 6.3±0.2; Holstein and Gir, respectively). The increase in LHR expression occurred in parallel to the previously described increase in intrafollicular estradiol concentrations at this same interval of follicular growth (Arashiro et al., 2013). Results of the present study demonstrate that LHR gene is expressed even in follicles smaller than the expected diameter at deviation in both *Bos taurus* and *Bos indicus* females. Also, relative LHR expression increases during the establishment of dominance, consistently with the progression of steroidogenesis. Further studies will evaluate whether and in which proportion LHR isoforms are present in these follicles.

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A058 Folliculogenesis, Oogenesis and Superovulation

### **The role of insulin-like growth factor-I on heat-induced microtubule changes and meiotic progression in bovine oocytes**

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**Keywords:** IGF-I, meiotic progression, microtubule.

Heat stress promotes, among several physiological and cellular alterations, changes in the reproductive tract microenvironment, compromising oocyte nuclear maturation, fertilization and embryonic development. It has been demonstrated that insulin-like growth factor-I (IGF-I) plays a thermoprotective role in the bovine oocyte. Therefore, the objective of this study was to: 1) determine the effect of heat shock on bovine oocyte microtubule and meiotic progression and 2) evaluate the thermoprotective role of IGF-I in this context. Cumulus-oocyte complexes (COCs) collected from slaughterhouse ovaries were subjected to control (38.5°C for 22 hours) and heat shock (41°C for 14 hours followed by 38.5°C for 8 hours) treatments in the presence of 0 or 100 ng/ml IGF-I during *in vitro* maturation (IVM). COCs were denuded, fixed in 3.7% formaldehyde and permeabilized in 0,5% Triton-X 100. Oocytes were incubated in bovine anti  $\alpha$ -tubulin mouse IgG monoclonal primary antibody (2 $\mu$ g/mL) followed by Alexa Fluor<sup>®</sup> 488 goat anti-mouse IgG (10 $\mu$ g/mL) and Hoechst 33342 (5 $\mu$ g/mL) to evaluate microtubules and meiotic progression, respectively. Microtubule fluorescence intensity was quantified in the metaphase plate, polar body and total oocyte using the software Image J 1.45s. Parametric data were subjected to least-squares analysis of variance and non-parametric data were analyzed by Wilcoxon's test of SAS. Heat shock reduced ( $59 \pm 0.11\%$  to  $29 \pm 0.11\%$ ;  $P < 0.05$ ) the percentage of metaphase II oocytes and reduced ( $P < 0.05$ ) metaphase plate microtubule organization ( $1.2 \pm 0.06$ , and  $1.4 \pm 0.06$  arbitrary units to control and heat shock, respectively). Addition of 100 ng/mL IGF-I to IVM medium minimized ( $P < 0.05$ ) the deleterious effect of temperature on oocyte meiotic progression increasing the percentage of MII of  $29 \pm 0.11\%$  to  $35 \pm 0.11\%$ . However, IGF-I did not affect microtubule organization in heat-shocked oocytes. There was no temperature or IGF-I effect on polar body microtubules organization. In conclusion, IGF-I played a thermoprotective role on meiotic progression. However, the same effect was not observed on microtubule organization. It has been shown that oocyte meiotic division is intimately linked to microfilament. Therefore, the positive effect of IGF-I on meiotic progression could be mediated by microfilament structure. Our previous studies demonstrated the thermoprotective effect of IGF-I on microfilament organization in bovine oocytes exposed to heat shock.



A059 Folliculogenesis, Oogenesis and Superovulation

### **Influence of melatonin and FSH in a sequential culture medium on *in vitro* development of isolated caprine preantral follicles**

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**Keywords:** cultive, immunohistochemistry, ovary.

Studies have demonstrated that melatonin is a potent antioxidant (Gao et al., 2012, J. Pineal. Res., 52, 305-311) and its receptors were expressed in granulosa cells of secondary and antral follicles in goats (Menezes et al., 2011, VI Jornada de Iniciação Científica, Univasf). One gonadotropin that has been widely used in *in vitro* studies for maintenance of viability and to promote follicular growth is FSH (Saraiva et al., 2010, Reprod. Sci. 17, 1135-1143). Recently, the use of Melatonin associated with FSH in the *in vitro* culture medium promoted an increase in the diameter of preantral follicles after culturing goat ovarian tissue for 7 days (Rocha et al., 2013, Domest. Anim. Endocrinol., 44, 1-9). However, the effect of melatonin, alone or associated with FSH, on the *in vitro* growth of preantral follicles isolated from caprine ovaries is not known. The aim of this study is to evaluate the effect of melatonin and FSH on the *in vitro* development of isolated goat preantral follicles. Goat ovaries (n=60) were collected in slaughterhouse and after mechanical isolation, preantral follicles (diameter  $\leq 200 \mu\text{m}$ ) were individually cultured in 100  $\mu\text{L}$  droplets, during 12 days, at 39°C, in  $\alpha$ -Minimum Essential Medium ( $\alpha$ -MEM<sup>+</sup>; GIBCO-Invitrogen, St Louis, EUA; control medium) alone or added by fixed concentrations of Melatonin (100 or 1000 pg/mL) or in sequential medium containing Melatonin (100 pg/mL: from day 0 to 6; 1000 pg/mL: from day 6 to 12), corresponding to Experiment 1. For the Experiment 2, follicles were cultured in control medium or sequential FSH medium (100 ng/mL: from day 0 to 6; 500 ng/mL: from day 6 to 12) or sequential melatonin associated with sequential FSH. Parameters such follicular survival and extrusion rate, antrum formation, recovery rate of *in vitro* grown oocytes (diameter  $\geq 110 \mu\text{m}$ ) were analyzed at each 6 days and data were submitted to Qui-square test. Follicular diameter and growth rate of Experiment 1 were submitted to Shapiro-Wilk and Tukey tests, and those from Experiment 2, to Kruskal-Wallis test ( $P < 0.05$ ). Results from Experiment 1 showed that, after 12 days of culture, sequential melatonin medium increased ( $P < 0.05$ ) the percentage of normal follicles and the recovery rate of *in vitro* grown oocytes, compared with control and 1000 pg/ml of melatonin. In Experiment 2, all treatments increased ( $P < 0.05$ ) the percentage of normal follicles, antrum formation and recovery rate of oocytes, compared with the control, however no differences were observed among treatments ( $P > 0.05$ ). In conclusion, this study demonstrated that the use of defined concentrations of Melatonin and/or FSH, added at specific periods of culture and in a progressive way, can be successfully used to preserve follicular survival and to promote goat oocyte growth.



A060 Folliculogenesis, Oogenesis and Superovulation

**Extract of *amburana cearensis* has cytotoxic effect on sheep preantral follicles preserved *in vitro***

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**Keywords:** *amburana cearensis*, apoptosis, ovary.

*Amburana cearensis* is a native medicinal plant from the semi-arid region in the northeast of Brazil (Caatinga biome) (Albuquerque e Oliveira, 2007, Journal of Ethnopharmacology, 113, 156–170), which has antibacterial and antioxidant actions (Bravo et al., 1999, Phytochemistry, 50, 71–74). However, there are no reports about this plant and ovine folliculogenesis. The aim of this study was to assess survival and apoptosis rates of ovine preantral follicles preserved *in vitro* in the extract of *A. cearensis*. Ovine ovaries (n=08) were collected in a slaughterhouse and divided into fragments (3x3x1 mm), one of them was fixed and used for histology (fresh control). The remaining fragments were preserved in Minimal Essential Medium (MEM – Sigma 87 Chemical Co, St Louis, USA; control medium) or different concentrations of the extract of *A. cearensis* (0,1; 0,2 or 0,4 mg/mL), at 4°C for 6 h. After conservation, the fragments were fixed and submitted to morphological analysis of the preantral follicles, which were classified as normal or atretic. Analysis of oocyte and granulosa cell apoptosis was performed using TUNEL, with the *in situ* cell death detection Kit (Roche Diagnostics Ltd., 158 Indianapolis, USA). The rates of follicular survival and apoptosis were analysed by Tukey and qui-square tests, respectively (P<0.05). After preservation in MEM, rates of follicular survival and apoptotic cells were similar (P>0.05) to those found in fresh control. However, preservation of ovarian tissue in different concentrations of the *A. cearensis* extract reduced (P<0.05) the percentage of normal follicles and increased (P<0.05) rates of follicular apoptosis, compared with fresh control and MEM. In conclusion, preservation of ovine ovarian tissue at 4°C for 6 h in the extract of *A. cearensis* increased apoptosis rate in preantral follicles, thus showing a cytotoxic effect.



A061 FTAI, FTET and AI

### Factors that influence the results after TAI protocols in Nelore and Nelore x Angus heifers inseminated between 12 and 14 months old

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**Keywords:** heifers, induction of puberty, TAI.

The response of heifers from different breed submitted to protocols for induction of puberty and TAI between 12 and 14 month old was evaluated. At the beginning of the induction of puberty protocol, Nelore heifers (NE; n=433) with  $11.4 \pm 0.05$  months (mo) old, weighted  $278.1 \pm 1.6$  Kg and presented a body condition score (BCS; 1 to 5 scale) of  $3.6 \pm 0.02$ ; and Nelore x Angus heifers (AN; n=414) with  $9.2 \pm 0.26$  mo old, weighted  $306.9 \pm 1.7$  Kg and presented a BCS of  $4.1 \pm 0.02$ . All heifers received a previously used for 27 days (4<sup>th</sup> use) intravaginal progesterone (P4) device (CIDR<sup>®</sup>; 1.9 g of P4, Zoetis) on D-22 (22 days before the onset of the synchronization protocol). On D-10, the CIDR was removed and all heifers received 0.5 mg of estradiol cypionate I.M. (ECP<sup>®</sup>, Zoetis). On D0, heifers were evaluated by transrectal ultrasonography (Aloka SSD 500) to detect the presence of corpus luteum (CL). All heifers had their ovulation synchronized with the following protocol: insertion of a previously used CIDR for 18 days (3<sup>rd</sup> use) and 2.0 mg I.M. of estradiol benzoate on D0; 12.5 mg I.M. of dinoprost tromethamine (Lutalyse<sup>®</sup>, Zoetis) on D7; on D9 the CIDR was removed and the heifers received 0.5 mg I.M. of ECP and 200 IU of eCG. The TAI was performed on D11, 48 h after CIDR withdrawal. Pregnancy diagnosis was performed on D41. Data were analyzed using GLIMMIX procedure of SAS. The analysis for the presence of CL on D0 included in the model the effects of age, breed, BCS and weight. To analyze pregnancy per AI (P/AI) it was included in the model the effects of age, breed, presence of CL on D0, AI technician and semen. The data were reported as least square means and significance was defined when  $P < 0.05$ . The presence of CL on D0 did not differ between breeds (NE: 79.9% and AN: 78.8%). Regardless breed, heavier heifers were more likely ( $P < 0.05$ ) to have CL on D0. There was an effect of presence of CL on D0 ( $P < 0.05$ ) for P/AI (With CL: 57.5% [370/650] vs. Without CL: 44.3% [74/169]). There was no interaction between presence of CL on D0 and breed affecting the P/AI. The NE heifers had lower ( $P < 0.05$ ) P/AI (46.9%; 199/433) than AN heifers (62.6%; 259/414). A significant interaction between presence of CL on D0 and weight was found for P/AI. In heifers with CL, the weight did not affect ( $P > 0.1$ ) P/AI. However, among heifers without CL, heavier heifers were more likely ( $P < 0.05$ ) to become pregnant. The BCS influenced the heifers response to induction of puberty protocols. In summary, heifers can be inseminated with younger ages; however, the presence of CL at the beginning of the TAI synchronization protocol and the BCS of heifers can determine the reproductive success. Furthermore, under the same conditions Nelore x Angus heifers have higher reproductive outcomes than Nelore heifers.



A062 FTAI, FTET and AI

### **Effect of the moment of insemination after progesterone device removal on conception rate in lactating dairy cows**

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**Keywords:** conception rate, insemination momento, TAI.

The aim of this trial was to determine whether delaying of the time to perform the timed artificial insemination (TAI) from 60 to 72 h after progesterone device removal affect the conception rate in lactating Holstein cows. The trial was conducted in three commercial dairies located in Cuenca Mar and Sierras region in the southeast of Buenos Aires province, Argentina [Herd 1 (n=72), Herd 2 (n=76) and Herd 3 (n=104 cows)]. The total milk production of 305 days, DIM and number of lactation were respectively: Herd 1 = 8,295±750; 170±88.4 e 2.6±1.3; Herd 2 = 8,069±1,050; 202.8±80 e 2.4±1.4; and Herd 3 = 8,264±1,021; 106±1.7 e 2.2±1.2. On the first day of the synchronization protocol (D0), cows received an intravaginal progesterone device (DIB, Syntex, Argentina) and 2 mg I.M. of estradiol benzoate (EB, Syntex, Argentina). On D7, the device was removed and 0.5 mg Clorprostenol (Cyclase, Syntex) I.M. was administered and all cows received 1 mg of EB 24 h later. After the last EB treatment, cows were randomly assigned in one of two groups (TAI60h and TAI72h), and were timed inseminated at 60 or 72 h after progesterone device removal. All cows received 10 ug of busereline acetate (Gonasyn, Syntex) at the same moment of the TAI. Pregnancy was diagnosed 32 days after TAI using transrectal ultrasonography. Data was analysed using PROC CATMOD of SAS. There was no effect (P>0.05) of TAI moment (60 and 72 hours) on the conception rate, which on the Herd 1 was 32.5% and 40.0%; on the Herd 2, 38.9% and 32.5%; and on Herd 3, 37.7% and 37.3%, respectively. In conclusion the timing to performe the TAI (60 or 72 h) after the progesterone device removal did not affect the conception rate of lactating dairy cows.



A063 FTAI, FTET and AI

### **Profile of progesterone releasing of intravaginal device (Vallée®) used for synchronization of ovulation protocols for TAI**

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**Keywords:** fertility, progesterone, synchronization of ovulation.

The current study evaluated the progesterone (P4) blood releasing of intravaginal devices containing 1.2 (DMax; Dispolcel Max<sup>®</sup>; Vallée S.A.) or 0.6g of P4 (DMonouso; Dispolcel Monouso<sup>®</sup>; Vallée S.A.). Also, it was tested the number of use of each device (new; 1X=used for 8 days or 2X=used for 16 days). Thirty six Holstein cows were allotted in six treatments groups (n=6 in each group): P4Control new (DIB<sup>®</sup>; MSD Saúde Animal); DMax new; DMax 1X; DMax 2X; DMonouso new e DMonouso 1X. Blood was collected at: 0, 6, 12, 18, 24, 48, 72, 96, 120, 168, 192, 198, 204 e 216 h after device insertion to quantify the circulating P4 concentration. Data were tested according to the normality of residues and homogeneity of variances using the Guided Data Analysis of the SAS. Posteriorly, data were analyzed by Proc GLIMMIX using repeated measures analysis. The averages of P4 concentrations were compared by LSmeans. Significance level was set at 5%. The profile of P4 releasing was similar among P4Control new Group ( $1.6 \pm 0.3^{ab}$  ng/mL) and new devices groups (DMax new= $1.9 \pm 0.1^a$  ng/mL; and DMonouso new= $1.6 \pm 0.1^{ab}$  ng/mL) or DMax 1X Group ( $1.2 \pm 0.9^{bc}$  ng/mL). Devices containing 1.2g previously used twice and 0.6 g used once showed lower concentration of P4 than control group (DMax 2X= $1.1 \pm 0.7^c$  ng/mL and DMonouso 1X= $1.0 \pm 0.8^c$  ng/mL). All groups presented similar pattern of P4 releasing curve, characterized by an elevation on the circulating P4 to maximum level at six hours after device insertion, which was maintained until the moment of device removal. The present data indicate that Dispolcel Max<sup>®</sup> 1.2g (new, previously used once or twice) or Dispolcel Monouso<sup>®</sup> 0.6g (new or previously used once) can release sufficient quantity of P4, enabling the use in timed artificial insemination programs in cattle. Acknowledgements: Apta Dairy Cattle, Nova Odessa-SP.



A064 FTAI, FTET and AI

### Different sources of equine chorionic gonadotropin (eCG) on follicular growth and ovulation rate of *Bos indicus* cattle

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**Keywords:** eCG, ovulation, synchronization of ovulation.

The objective of this study was to evaluate the effect of different sources of equine chorionic gonadotropin (eCG) on the dominant follicle growth and ovulation rate of lactating beef cows submitted to synchronization of ovulation program. Forty-eight lactating *Bos indicus* cows (multiparous with 30 to 45 days postpartum) with body condition score greater than 2 (1 to 5 point scale) were used. At random stage of the estrous cycle (D0), cows received 2mg of estradiol benzoate (Sincrodiol<sup>®</sup>, Ourofino, Brazil) and an intravaginal progesterone device (Sincrogest<sup>®</sup>, Ourofino, Brazil). On the same day, an ultrasonographic examination (US; Aloka SSD500) was performed to verify the absence of corpus luteum (only cows without CL were used). At the P4 device removal (D8), cows were submitted to the US examination to measure the diameter of the larger follicle, and then were homogeneously allocated in one of three treatment groups [SincroeCG Group; Positive Control Group (PC Group) and Negative Control Group (NC Group)] according to follicular size. On the same day, the cows received 500µg of PGF<sub>2α</sub> (Sincrocio<sup>®</sup>, Ourofino, Brazil) and 1.0 mg of estradiol cypionate (ECP<sup>®</sup>, Zoetis, Brazil). Cows from PC Group received 300IU of eCG (Folligon, MSD<sup>®</sup>, Netherland) and cows from SincroeCG Group received 300IU of eCG (SincroeCG<sup>®</sup>, Ourofino, Brazil). The animals from NC Group did not receive any additional treatment. On D10 and D12, US were accomplished to evaluate the follicular growth (mm/day) and ovulation rate (%). Data were analyzed by GLIMMIX of SAS. There was a treatment effect (P=0.02) on dominant follicle growth (0.85±0.22<sup>b</sup> mm/day on NC Group; 1.66±0.29<sup>a</sup> mm/day on PC Group and 1.80±0.23<sup>a</sup> mm/day on SincroeCG Group) and on ovulation rate [NC Group=50% (8/16)<sup>b</sup>; CP Group=87.5% (14/16)<sup>a</sup> and SincroeCG Group=81.3% (13/16)<sup>a</sup>; P=0.04]. In conclusion, both eCG sources are efficient to improve the final dominant follicle growth and ovulation rate in anestrous lactating beef cows submitted to synchronization of ovulation.



A065 FTAI, FTET and AI

### Conception rate in *Bos indicus* beef cows submitted to TAI protocols using a new or previously used progesterone devices, and different body condition scores

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**Keywords:** body condition score, estrus synchronization, insemination.

This study aimed to verify the effects of the number of uses of intravaginal progesterone devices and body condition score (BCS) on conception rates of *Bos indicus* beef cows submitted to timed artificial insemination (TAI) protocols. Thus, 1,122 multiparous Nelore cows were subjected to a TAI protocol using new or second and third use intravaginal progesterone device. All females were raised on pasture management in a farm in southern of Para state. At the beginning of the synchronization (D0), cows received an intravaginal progesterone device (DIB® 1.0 g of P4; Intervet/Schering-Plough) and 2 mg intramuscular (IM) of estradiol benzoate (EB; Gonadiol®, Intervet/Schering-Plough); on D8, the intravaginal device was removed and 300 IU of eCG IM (Folligon®, Intervet/Schering-Plough), 7.5 mg of PGF2 $\alpha$  (Veteglan Lueolítico®; Hertape Calier Animal Health), and 1 mg of EB (Gonadiol®, Intervet/Schering-Plough) were administrated. The TAI was performed 48 hours after progesterone device removal. Cows were categorized according to their BCS at the first day of synchronization using a 1 to 5 scale (Freitas J. R. Bras. Zootec. 37, 116-121). To analyze the effect of BCS on pregnancy success, cows were classified into one of two groups: 2.0 to 2.5 or 2.75 to 4.0 BCS. The data were analyzed by ANOVA, using the Statistical Analysis System version 9.2. Difference was considered when  $P < 0.05$ . Overall conception rate was 61.1% (573/1,122). There was a significant effect ( $P < 0.0001$ ) of number of uses of the intravaginal device on the conception rate [New = 60.00%<sup>a</sup> (198/330), Second use = 51.71%<sup>b</sup> (227/439) and Third use = 41.93%<sup>b</sup> (148/353)]. Females with BCS between 2.75 and 4.0 showed higher ( $P < 0.0001$ ) conception rate (69.75%<sup>a</sup>; 385/552) than cows with BCS between 2.0 and 2.5 (32.98%<sup>b</sup>; 188/570). It was concluded that the use of new intravaginal devices resulted in greater conception rates than previously used progesterone devices in synchronization of ovulation programs for TAI. Also lower conception rate was obtained when the TAI protocols were applied in low BCS cows (i.e BCS  $\leq$  2.5).



A066 FTAI, FTET and AI

### Plasma progesterone concentration and luteolysis rate in crossbred females at different stages of the estrous cycle

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**Keywords:** bovine, cloprostenol, luteolysis.

Induction of luteolysis using analogs of prostaglandin F2 $\alpha$  (PGF2 $\alpha$ ) has a fundamental role to synchronize estrous cycle in oocyte and embryo donors and embryo recipients. It is also essential in protocols for timed artificial insemination. Besides the presence of a functional corpus luteum, the efficiency of the luteolytic process varies according with the phase of the estrous cycle when PGF2 $\alpha$  is administered. (Fernandes, 2007. Rev. Bras. Reprod. Anim. 31, 406-414). The aim of the study was to compare the changes in progesterone (P4) concentration and the efficiency of luteolysis when PGF2 $\alpha$  was administered at different stages of the estrous cycle in bovine crossbred females. The study was conducted in the southern of Minas Gerais State, a region with mesothermic climate. Thirty crossbred cows and 30 crossbred heifers between the 6th and 17th days of the estrous cycle (estrus = day 0) were homogeneously allocated to one of three groups according to the period of the estrous cycle. P1: between the 6th and 9th days, P2: between the 10th and 13th days, and P3: between the 14th and 17th days. Animals were kept in pasture (*Brachiaria brizanta*). They received 0.5 mg of Sodic Cloprostenol (Sincrosin®, Vallée, São Paulo, Brazil) I.M. Blood samples were collected immediately before PGF2 $\alpha$  administration and then 36 hours after. The concentration of progesterone (P4) was evaluated by RIA. Luteolysis was considered effective if the difference between the two analyses of P4 was greater than 75%. The concentration of P4 between the initial and final periods (ANOVA) and the effectiveness of luteolysis between periods and between animal categories (Fisher Test) were compared. Differences were considered at the 5% level. There was no effect of category (cow or heifer) in the studied variables. The mean initial concentrations of P4 did not differ between the three periods of the estrous cycle ( $2.42 \pm 1.02$ ,  $2.77 \pm 1.25$  and  $2.18 \pm 0.62$  ng/mL), as well as P4 concentrations 36 hours after PGF2 $\alpha$  administration ( $0.29 \pm 0.16$ ,  $0.34 \pm 0.18$  and  $0.26 \pm 0.15$  ng/mL) for P1, P2 and P3, respectively. Also no difference was found in luteolysis rate: P1 = 80.0% (17/20); P2 = 90.0% (18/20) and P3 = 100.0% (20/20;  $P > 0.05$ ), showing that the PGF2 $\alpha$  was similarly effective in all periods of the estrous cycle, regardless of the animal categories. We concluded that the treatment with 0.5 mg of Sodic Cloprostenol is effective to induce luteolysis in different periods of the estrous cycle of crossbred cows and heifers.

Acknowledgments: Fapemig.



A067 FTAI, FTET and AI

### Fertility of lactating dairy cows submitted to TAI protocols with GnRH or estradiol compared to a pre-synchronization or AI after estrus detection

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**Keywords:** artificial insemination, bovine, fixed-time.

In recent years, there has been a significant reduction in conception rates of dairy cows. This reduction is apparently linked to the increase in milk production per cow, resulting in drastic decrease in estrus expression, and consequently the efficiency of conventional artificial insemination (AI). Therefore, more effective timed AI (TAI) protocols have been developed. Thus, the first aim of this study was to compare the conception rates of TAI protocols based on GnRH vs. estradiol benzoate (EB) associated with an intravaginal progesterone (P4) device on the first day of the protocol (D0). The second objective was to compare the protocols mentioned to one that includes a pre-synchronization, but without P4. The third objective was to compare the three TAI protocols vs. AI after estrus detection. Between 41 and 47 d postpartum, 789 cows were randomly divided into four groups: G1 (n=187) pre-synchronization with PGF2 $\alpha$  on D-8 and GnRH on D-6, followed by synchronization with GnRH on D0, two injections of PGF2 $\alpha$  on D7 and D8, EB on D8, and TAI 36 h later; G2 (n=215) synchronization with EB+P4 on D0, P4 withdrawal on D8 and other procedures were identical as in G1; G3 (n=192) synchronization with GnRH+P4 on D0, P4 withdrawal at D8 and other procedures same as G1; and G4 (n=195): AI after estrus detection induced by two PGF2 $\alpha$  treatments 14 d apart. Cows not observed in estrus were treated with GnRH and PGF2 $\alpha$  at 7 and 14 d after the second PGF2 $\alpha$ , respectively. All injections were I.M.. Transrectal ultrasonographic examination was performed 30 d after AI to evaluate the conception rates. Statistical analysis was performed by logistic regression using PROC GLIMMIX of SAS. The first comparison between G2 and G3 showed higher (P=0.02) conception rates in G2 (42.8%) compared to G3 (32.3%). When the comparison was between G1 vs. G2+G3, G1 (44.4%) and G2+G3 (37.8%) were not different (P=0.13). Finally, conception rates of G4 (55.2%; 64/116) when only bred cows were considered in the analysis, were higher (P=0.002) than the conception obtained by all TAI groups combined (39.9%). However, when considering pregnancy out of all the cows available for G4 (64/195), pregnancy rate (32.8%) tended to be lower (P=0.07) than in TAI groups (39.9%). It is concluded that a EB+P4-based TAI protocol for first service in lactating cows presented better results than the GnRH+P4-based protocol. It can also be concluded that, because no difference was detected in relation to the pre-synchronization strategy in fertility, the use of EB+P4 was more effective by allowing AI earlier. In addition, although the TAI protocols of this trial had lower conception rates than AI after estrus detection, they resulted in a better pregnancy rate.

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A068 FTAI, FTET and AI

### Fertility of Nelore females with different antral follicles counts in a FTAI program

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**Keywords:** antral follicle population, *bos taurus indicus*, conception rate.

The objective of this study was to compare conception rates between Nelore females with different categories of antral follicle count (AFC) submitted to a protocol for fixed-time artificial insemination (FTAI). A total of 481 Nelore multiparous cows aging  $6.7 \pm 3.6$  years, with body condition score (scale 1-5) of  $2.7 \pm 0.4$  and postpartum interval of  $92.0 \pm 49.9$  days were submitted to a protocol for synchronization of the follicular wave emergence and ovulation beginning on random day of the estrous cycle (D0). At this moment, cows received an intravaginal progesterone releasing device (Sincrogest<sup>®</sup>, Ouro-Fino, São Paulo, Brazil), associated with an intramuscular injection of 2.0mg of estradiol benzoate (Sincrodiol<sup>®</sup>, Ouro-Fino, São Paulo, Brazil). On D8, the progesterone devices were removed and cows were treated with 300 I.U. of equine chorionic gonadotropin (eCG; Novormon<sup>®</sup>, MSD Saúde Animal, São Paulo, Brazil) I.M., 500 $\mu$ g of cloprostenol (Sincrocio<sup>®</sup>, Ouro Fino, São Paulo, Brazil) I.M. and 1.0 mg of estradiol cypionate (ECP<sup>®</sup>, Pfizer, São Paulo, Brazil) I.M.. On D10, the FTAI was performed. The antral follicular population was assessed on D4 of the synchronization protocol by ultrasonography using a 8.0 MHz linear transducer (Pie-Medical, Falco 100, Maastricht, Holanda), antral follicles with diameter  $\geq 3$ mm were counted in both ovaries, and cows were grouped into three categories according to the AFC: low ( $\leq 34$  follicles), intermediate (between 34 to 53 follicles) and high AFC ( $\geq 53$  follicles). Pregnancy diagnosis was performed 55 days after the TAI by transrectal ultrasonography. Conception rates among AFC groups were compared with the chi-square test ( $P < 0.05$ ). The overall conception rate was 29.3% (141/481). There was no statistical difference on conception rates among the AFC categories (Low=29.34%;49/167; Intermediate=29.33%;44/150, and High=29.27%;48/164). These results suggest that the AFC does not affect conception rates following FTAI programs. Nevertheless, the use of eCG in synchronization protocol may have minimized the influence of AFC on reproductive outcome in this study. Further studies are necessary to cover the issue in FTAI protocols without the use of this drug, as well as in artificial insemination upon estrus detection programs.



A069 FTAI, FTET and AI

### **Productive aspects and beta-hydroxybutyrate concentrations of high production Holstein cows, diagnosed as pregnant or non pregnant until 150 days in milk**

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**Keywords:** beta-hidroxibutyrate, conception, dairy herd.

The aim of the present study was to evaluate the differences between milk production and blood beta-hydroxybutyrate (BHB) concentration of high producing Holstein cows that become or not pregnant until 150 days in milk (DIM). It was used a database of biochemical and productive measures of 250 females (121 primiparous and 129 multiparous) from a commercial farm (Agrindus S/A). Body condition score (BCS) and milk production were recorded at calving (D0) and at 30, 60 and 90 DIM. Blood concentrations of beta-hydroxybutyrate (BHBA) were analyzed using a handheld meter Precision XTRA<sup>®</sup> at calving (D0), 15 and 30 DIM. After 60 days of voluntary waiting period, cows were artificially inseminated upon detection of estrus. The interval from calving to first service was recorded and the pregnancy diagnosis was performed 45 days after insemination by transrectal palpation. Retrospectively, cows were grouped according to the 150 DIM pregnancy status (130 pregnant and 120 non pregnant cows) and the averages of BCS, milk production, BHBA and interval calving-first service were compared. All variables were analyzed by the GLIMMIX procedure of SAS<sup>®</sup>. The statistical models included the classificatory variable pregnancy at day 150 – and, linearly, by BCS (0, 30 and 90 DIM), milk production (30, 60 and 90 DIM) and BHBA (0, 15 and 30 DIM) variables. The data are presented as average ± standard error mean (SEM), considering P<0.05. There was no difference between pregnancy status among the different time points on BCS (0 - 3.16±0.01; 30 - 2.91±0.01; 60 - 2.90±0.01 and 90 DIM - 2.90±0.01) and milk production (30 - 37.6±0.5 liters; 60 - 39.8±0.4 liters and 90 DIM - 40.8±0.5 liters). Regardless pregnancy status at 150 DIM, the concentrations of BHBA were similar both at calving (0.42±0.01 mmol/L) and 60 DPP (0.73±0.06 mmol/L). However, pregnant cows had lower blood BHBA concentrations at day 15 (0.62±0.07 vs. 0.82±0.09 mmol/L; P=0.04) and shorter calving/first service interval (65.3±1.7 vs. 76.2±2.9 DPP; P=0.02) than non pregnant cows. Therefore, it was concluded that high production Holstein cows that become pregnant until 150 DIM have lower blood concentrations of BHBA at 15 DIM and a shorter calving/first service interval than non pregnant cows until this period.



A070 FTAI, FTET and AI

### Seasonal variation in pregnancy rate and weight gain in embryo recipient maintained in pasture condition

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**Keywords:** bovine, nutrition, recipients.

The pregnancy rate of recipients has the greatest impact on the cost of a commercial embryo transfer program in cattle (VARAGO, et al., 2008. Rev. Bras.Reprod. Ani, 32, 100-109). The aim of this study was to evaluate the seasonal weight gain and the effect on pregnancy rate in crossbred embryo recipients maintained in pasture condition. It was conducted in a farm located in the Southwest of Minas Gerais State - Brazil. The region has climate classification CW, according to Koepen. A total of 484 *in vitro* produced (IVP) embryos from Gyr donors using sexed sorted semen were used. Heifers have their estrus synchronized using intramuscular injection of 0.530mg of cloprostenol. The recipients were weighed on the same day of the embryo transfer and again at 23 to 25 days later, when the pregnancy diagnosis was performed by ultrasonography. According to the daily weight gain, heifers were divided into four groups: G1 (n=132): below 150g, G2 (n=133): from 151 to 250g; G3 (n=119): from 251 to 350g and G4 (n=100): above 350g. After embryo transfer, the recipients were kept on *Brachiaria brizanta* pasture with water and mineral supplementation *ad libitum*. It was compared the pregnancy rate among the different weight gains and the proportion of heifers in each weight gain group in two seasons (compared by Fisher test): P1 – rain season (November to April), with a stocking rate of 1.3 animal unit/ha and P2 – dry season (May to October), with a stocking rate of 1.05 AU/ha. In the rain season, there was equitable distribution of the recipients among the different weight gain groups (P>0.05). However, during dry season, it was observed a greater frequency (P< 0.05) of heifers presenting lower daily weight gains (i.e. below 250g). In this last season, different (P<0.05) distribution (%) of heifers according to the daily weight gain was found (G1=33.2<sup>a</sup>, G2=31.4<sup>a</sup>, G3=21.8<sup>b</sup> and G4=13.6<sup>c</sup>). There was no difference between seasons on pregnancy rates (Dry season = 42.3 vs Rain season 45.8%). Recipientes that presented greater weight gain (i.e. above 250g G3= 52.1% and G4= 51.0%) showed higher (P<0.05) pregnancy rate than those with lower weight gain (G1= 35.6% and G2 = 40.6%). For the same weight gain, there were no differences between seasons (P>0.05). It was concluded that weight gain is an important parameter to be considered in the management of IVP embryo recipients. Furthermore, the reduction in the stocking rate during the dry season is not sufficient to avoid the impact of weight gain on the pregnancy rates of the crossbred embryo recipients.

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A071 FTAI, FTET and AI

### **Comparison between the animal category on the occurrence of estrus, ovulatory follicle diameter and conception rate in Nelore cattle**

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**Keywords:** *Bos indicus*, estrus detection, TAI.

The aim of this study was to compare the effect of animal category on the occurrence of estrus, on the ovulatory follicle diameter and on conception rate of females submitted to a fixed-time artificial insemination (FTAI) protocol. A total of 200 female Zebu, with a body condition score of  $3.19 \pm 0.42$  (scale 1-5) and divided according to animal category in lactating multiparous cows ( $n = 141$ ;  $64.58 \pm 18.42$  days postpartum) and heifers ( $n = 59$ ). Prior to the beginning of the synchronization protocol, heifers were submitted to clinical gynecological examination using ultrasound and only pubertal heifers (presence of corpus luteum) were used. On a random day of the estrous cycle (D0), the animals received an intravaginal progesterone (P4) device (PRIMER<sup>®</sup>, Tecnopec, São Paulo, Brazil) associated with 2.0mg estradiol benzoate (RIC-BE<sup>®</sup>, Tecnopec, São Paulo, Brazil) I.M.. On D8, the intravaginal P4 device was removed and 300 IU of equine chorionic gonadotropin (Folligon<sup>®</sup>, Intervet, São Paulo, Brazil), 150µg of d-cloprostenol (Prolise<sup>®</sup>, Tecnopec, São Paulo, Brazil) and 1.0 mg of estradiol cypionate (ECP<sup>®</sup>, Pfizer, São Paulo, Brazil) were intramuscularly administered. The animals were then marked with a chalk maker (RAIDEX<sup>®</sup>, Walmur, Porto Alegre, Brazil) between the sacral tuberosity and the tail insertion. On D10, the animals were categorized into two groups according to the occurrence of estrus: Group 1 (G1) - No estrus, presence and permanence of color ink intensity ( $n=45$ ); Group 2 (G2) - intermediate estrus, loss of ink color intensity ( $n=38$ ) and Group 3 (G3) - estrus, complete removal of ink ( $n=117$ ). Immediately after, the diameter of the ovulatory follicle (DFOL) was measured by transrectal ultrasonography using a 5.0MHz linear transducer (Mindray, DP2200vet, São Paulo, Brazil) and FTAI was performed in all animals. Pregnancy diagnosis was performed by ultrasonography 45 days after FTAI. The program SPSS (version 19) was used to perform the statistical analysis ( $P < 0.05$ ). The overall conception rate was 49.0% (98/200). There was no significant difference between the occurrence of estrus and animal category in the experimental groups. The occurrence of estrus among groups was 46.0% (G1-cows), 54.0% (G2-cows), 33.3% (G1-heifers) and 66.7% (G2-heifers). Regarding DFOL, it was found that cows have a greater diameter ( $12.51 \pm 2.98$ mm) than heifers ( $9.54 \pm 3.61$ mm). Furthermore, there was statistical difference ( $P=0.01$ ) on the conception rate between the animal categories (Cows=54.6%, 77/141 and heifers=35.6%, 21/59). It is possible to conclude that the animal category did not influence the occurrence of estrus; however, cows have greater ovulatory follicle diameter and greater conception rates than heifers. Furthermore, the occurrence of estrus is not a good parameter to predict the ovarian follicular and pregnancy response to FTAI protocols in heifers.



A072 FTAI, FTET and AI

### **Follicular diameter at insemination moment as a predictor of ovulatory response and pregnancy success in dairy buffaloes submitted to TAI**

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**Keywords:** fertility, ovulatory follicle, ultrasonography.

This study aimed to establish the probability of ovulation and pregnancy per AI (P/AI) through the diameter of the largest follicle at the moment of artificial insemination (AI) of lactating buffaloes subjected to timed artificial insemination (TAI) protocols during the nonbreeding season (spring-summer). Thus, it was measured the diameter of the largest follicle at the moment of TAI ( $\phi$ FolAI) of 175 dairy buffaloes raised in six farms of the Ribeira Valley, State of São Paulo. All females were submitted to the same synchronization protocol. On D-12 all animals received a new or used once or twice intravaginal progesterone (P4) device (1 g of P4; Sincrogest®, Ourofino Agronegócio) plus 2.0 mg I.M. of estradiol benzoate (Sincrodiol®, Ourofino Agronegócio). On D-3 (PM), females received I.M. 0.53mg of PGF2a (Cloprostenol, Sincrocio®, Ourofino Agronegócio) and 400 IU of eCG (Novormon®, MSD Saúde Animal), followed by P4 device removal. On D-1 (PM), 10 $\mu$ g I.M. of buserelin acetate (GnRH, Sincroforte®, Ourofino Agronegócio) were administrated. The TAI was performed 16 hours after GnRH treatment (D0; am). A single bull was utilized for all inseminations. Ultrasound exams (Chison D600Vet, China) were performed to evaluate the  $\phi$ FolAI (mm), occurrence of ovulation rate (CL presence at day 10) and pregnancy 30 days after TAI. The prediction of the occurrence of ovulation (Ov) and pregnancy (P30) relative to  $\phi$ FolAI was obtained by the regression  $Logit = intercept + slope * (\phi FolAI)$  calculated through the solution *Interactive Data Analysis* of SAS®. The probability could be obtained by the formula:  $[EXP(Logit)/1+EXP(Logit)] * 100$ . The adjusted *odds ratio* for the risk of pregnancy 30 days after AI relative to the classes of  $\phi$ FolAI were evaluated (<12.0; 12.0 to 15.0; and >15.0 mm). Variables were considered different when  $P < 0.05$ . The overall means observed were  $\phi$ FolAI - 13.3 $\pm$ 0.2 mm; ovulation rate - 84.0% (147/175); and pregnancy rate on D30 - 67.4% (118/175). The logistic curves obtained were:  $Logit (Ov) = -5.71 + 0.60 * \phi FolAI$  ( $P < 0.0001$ ); and  $Logit (P30) = -2.23 + 0.22 * \phi FolAI$  ( $P = 0.0007$ ). Pregnancy rates and adjusted *odds ratio* (confidence interval) for P30 in each class of  $\phi$ FolAI were: <12.00 mm - 46.3% (25/54)<sup>b</sup> - reference; 12.01 to 15.00mm - 77.0% (57/74)<sup>a</sup> - 3.889 (1.807 to 8.372) and >15.00 mm - 76.60% (36/47)<sup>a</sup> - 3.796 (1.594 to 9.039)  $P = 0.0007$ . It was concluded that the  $\phi$ FolAI can be consider a predictor to ovulatory responses and pregnancy success in dairy buffaloes submitted to TAI synchronization protocol during nonbreeding season, in which females that had larger follicle at TAI are associated to higher ovulation and pregnancy rates.

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A073 FTAI, FTET and AI

### **The use of a GnRH analog at the time of the artificial insemination in dairy cattle**

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**Keywords:** bovine, buserelin acetate, conception rate.

The GnRH belongs to a group of decapeptides isolated from the hypothalamus, acting as a controller of the anterior pituitary gland, which has influence on reproductive processes mainly by regulating the release of follicle stimulating hormone and luteinizing hormone, which regulates the folliculogenesis. This function has been used for veterinary purposes, including the induction of ovarian activity, optimizing fixed-time artificial insemination, ovulation induction, and in the treatment of follicular ovarian cysts in cattle. The present study aims to evaluate the influence of GnRH analogue buserelin acetate (Sincroforte<sup>TM</sup>, Ouro Fino, Cravinhos, SP, Brazil) applied at the time of artificial insemination in dairy cows (range of milk production from 10 to 15kg per day). A total of 92 animals (Gir, Girolando, Holstein and Jersey) were randomly assigned into two groups. Group I (n=48) received 0.01 mg of buserelin acetate, IM at the time of AI and Group II (n=44) received saline solution, IM at the time of AI. All cows were inseminated 12 hours after detection of estrus by the same technician, using semen obtained from certified company. Pregnancy diagnosis was performed at 40 days after the AI by transrectal palpation performed by trained veterinarian. Data were analyzed by Chi Square, with 5% of significance level. The conception rate in group I was 54.17% (26/48), and in group II was 65.91% (29/44). No significant difference was found between groups ( $p > 0.05$ ).



A074 FTAI, FTET and AI

### Progesterone supplementation with 2 CIDR in lactating Holstein cows without corpus luteum submitted to E2/P4 based protocol

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**Keywords:** FTET, progesterone, TAI.

The objective was to evaluate the effect of the utilization of two intravaginal progesterone devices (CIDR®, Zoetis, SP, Brazil) in lactating Holstein cows without a corpus luteum (CL) at the beginning of the synchronization of ovulation protocol, on pregnancy per AI (P/AI) and pregnancy per ET (P/ET). This experiment was performed in 9 farms, 5 of them destined to fixed time embryo transfer (FTET; n=534) and 4 of them to timed AI (TAI; n=433). The protocol used was: D-10 2mg IM EB (2.0mL de Estrogin®, Farmavet, SP, Brazil) + CIDR (1.9g of P4); D-3 PGF 25mg IM (5.0 mL de Lutalyse®, Zoetis, SP, Brazil); D-2 removal of the CIDR + 1mg IM de ECP (0.5 mL, ECP®, Zoetis, SP, Brazil); D0 TAI or D7 FTET. In the D-10, cows were randomly assigned to receive 1 CIDR (CON) or 2 CIDR (2CIDR). The diameter of the ovulatory follicle was evaluated by ultrasound (US) in D0. Blood samples were collected for P4 measure on D-10, D-3 and D7, being considered synchronized animals with P4 ≥1.0 ng/mL in D7 (TAI) or CL visible in the US D7 (FTET). Pregnancy diagnosis was performed by US at 32d to verify the presence of the embryo and at 60d for the presence of the fetus. For evaluate binomial variables was used PROC GLIMMIX and continuous variables PROC MIXED. It was considered significant when P <0.05 and tendency if P <0.10. The P4 on D0 did not differ between groups (CON = 0.81 ± 0.09 ng/mL and 2CIDR = 0.88 ± 0.09 ng/mL). On the D-3 2CIDR group had higher P4 concentration (2.05 ± 0.22 ng/mL) compared to the CON group (1.65 ± 0.22 ng/mL). On D7, P4 of the CON (2.76 ± 0.16 ng/mL) was higher than the 2CIDR group (2.35 ± 0.16 ng/mL). The diameter of the ovulatory follicle (CON = 15.6 ± 0.36 mm and 2CIDR = 15.2 ± 0.37 mm) and ovulation (CON = 82.8% and 2CIDR = 81.0%) did not differ between treatments. The P/AI was similar at 32d (CON = 38.7% x 2CIDR = 42.1%) and 60d (CON = 32.9% x 2CIDR = 37.4%). In synchronized cows there was no difference between treatments on P/AI at 32d (CON = 42.6% x 2CIDR = 50.0%); however, the 2CIDR group tended to have higher P/AI at 60d (CON = 36.5% x 2CIDR = 45.8%). The P/ET did not differ between treatments at 32d (CON = 29.2% x 2CIDR = 32.2%) and 60d (CON = 24.3% x 2CIDR = 25.8%). The pregnancy loss did not differ between treatments in AI (CON = 14.4% x 2CIDR = 8.7%) and ET (CON = 17.9% x 2CIDR = 20.8%). The use of two P4 devices in lactating cows without CL increased P4 concentration during follicular growth and tended to increase P/AI in synchronized cows, but did not affect P/ET, suggesting that the concentrations of P4 can improve the P/AI due to improvement in oocyte quality.



A075 FTAI, FTET and AI

### **Efficiency of anticipation of the application of prostaglandin F<sub>2α</sub> in the Ovsynch protocol for ovulation synchronization and TAI in buffalo heifers during the breeding season**

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**Keywords:** buffalo heifers, Ovsynch, ovulation.

The present study aimed to evaluate the efficiency of the application of prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) twenty-four hours before the recommended period for *Ovsynch* protocol. It is known that buffalo heifers present three waves of follicular growth and it was believed that the administration of PGF<sub>2α</sub> 24h earlier could increase ovulation rate due to anticipated *turnover* compared to animals of two waves of follicular growth. Previous study demonstrated that buffalo heifers subjected to 24h in advance at application of PGF<sub>2α</sub> showed numerically greater ovulation rate at the end of the *Ovsynch* protocol (Gimenes *et al.*, 2005, In: 3rd Congresso Nazionale sull Allevamento del Bufalo and the 1st Buffalo Symposium of the Europe and the Americas, 238). Therefore, it could be expected to obtain higher conception rate in heifers that received this treatment. Thus, a total of 160 buffalo heifers homogeneously distributed into two groups (Control Group - GPGFD7 and PGF<sub>2α</sub> anticipated Group - GPGFD6), according to ovarian activity, the body condition score and the age were used in this study. The heifers from GPGFD7 group received intramuscularly (im) the application of 10mg of GnRH (Sincroforte<sup>®</sup>, Ourofino Agronegócio) at random stage of the estrous cycle (D-1) and the animals from GPGFD6 group received the same hormone on D0. On D6, all heifers received im 0.53mg of PGF<sub>2α</sub> (Cloprostenol sodium, Sincrocio<sup>®</sup>, Ourofino Agronegócio). On D8, 10mg of GnRH (Sincroforte<sup>®</sup>, Ourofino Agronegócio) was intramuscularly administered, and heifers were inseminated 16 hours after the application of the GnRH (D9). Ultrasound evaluations (Mindray DP2200Vet, China) were performed on D -1 to verify the ovarian activity and on the D39 for the pregnancy diagnosis. The data were analyzed using the GLIMMIX procedure of SAS<sup>®</sup>. The results obtained showed that there was no difference on the conception rate [35.9% (28/78) vs. 34.1% (28/82); P > 0.05] between the experimental groups GPGFD7 and GPGFD6, respectively. It is concluded that reducing 24 h of the interval between the applications of the first GnRH and PGF<sub>2α</sub> of the *Ovsynch* protocol did not improve the conception rate in buffalo heifers submitted to TAI during the breeding season.



A076 FTAI, FTET and AI

## Ovarian and endocrinology responses in Taurus and Zebu heifers submitted to different nutrition challenges

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**Keywords:** Holstein, Nelore, progesterone.

The aim of this study was to evaluate circulating progesterone concentration (P4) and ovarian follicular dynamics in *Bos indicus* and *Bos taurus* heifers under high (ACMS; weight gain of 900g per day) and low (BCMS; maintenance, NRC 2001) consumption of dry matter/energy. Cycling Holstein (n=16) and Nelore (n=16) heifers were used in a 2x2 factorial arrangement (cross-over). The experimental diet was given during 32 days (15 days before and 17 days during the hormonal protocol). The animals were pre-synchronized with two applications of cloprostenol (0.53mg, i.m. PGF<sub>2α</sub>, Sincrocio<sup>®</sup>, Ourofino Agronegócio) 12 days apart. To ensure that the progesterone from device is the unique source of P4, two doses of PGF<sub>2α</sub> were given 18 and 12 hours before device insertion. At onset of synchronization protocol (D0), heifers received a new intravaginal P4 device (CIDR<sup>®</sup>, Zoetis Brasil), 2mg of estradiol benzoate i.m. (BE, Sincrodiol<sup>®</sup>, Ourofino Agronegócio) and a dose of PGF<sub>2α</sub>. After 8 days, the P4 device was removed and 1mg of BE was administered 24 hours afterward. Ultrasonographic exams were performed every 24 hours during P4 device treatment (D0 until D8), every 12 hours from P4 device removal until ovulation and on D17 to evaluate the corpus luteum (CL) volume (mm<sup>3</sup>). Blood samples were collected daily from D0 to D10. The results were analysed using PROC MIXED of SAS 9.2 and presented as mean ± standard error (SEM). The new follicular wave emergence occurred at 3.3±0.1d, regardless of the breed and diet (P=0.22). Regardless of diet (P=0.93), number of recruited follicles were higher in Nelore than in Holstein heifers (46.9±4.1 vs. 26.3±3.5, respectively; P<0.001). Breed and diet affected the ovulatory follicle size (P=0.04 and P=0.0002) and CL volume (P<0.001 and P=0.002), respectively. The maximum diameter of ovulatory follicle was higher in Holstein heifers (14.9±0.7 and 12.7±0.5) than in Nelore heifers (13.4±0.4 and 11.6±0.4) receiving ACMS and BCMS diet, respectively. Furthermore, Holstein heifers had larger CL volume (6525.3±502.8 and 4653.4±503.8) than Nelore heifers (4188.1±531.7 and 2698.4±252.5). Regardless of the breed (P=0.29), heifers that received ACMS diet had greater follicular growth rate than heifers receiving BCMS diet, respectively (1.4±0.1 and 1.2±0.1 for Holstein; 1.3±0.1 and 1.1±0.1 for Nelore, P=0.002). The circulating P4 concentrations were greater in Nelore than in Holstein from D2 to D9 of protocol (P=0.001) and heifers receiving BCMS than those receiving ACMS from D1 to D8 (P=0.0002). Regardless of the breed, higher dry matter consumption during the hormonal protocol resulted in a greater follicular growth rate, a larger ovulatory follicle and a larger CL. (Preliminary data).

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A077 FTAI, FTET and AI

## Impact of the moment of TAI and rumen protected fat supplementation for cows submitted to early weaning in native grasslands in the pantanal

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**Keywords:** postpartum supplementation, production system, TAI moment.

The Pantanal is a biome with a natural aptitude for beef cattle due to the fields of native grasslands, however the herds' reproductive rates are low due to the variability of the diet (composition and availability of forage) and the physical and structural characteristics of farms. Alternatives such as food supplementation, fixed time artificial insemination (FTAI) and early weaning of calves can be used as strategies to increase reproductive efficiency of livestock. Thus, two experiments were conducted in fields of native pastures during the breeding season of 2012/2013, to assess: a) Experiment 1 - The effect of supplementation of postpartum cows on pregnancy rate to TAI and late breeding season (BS) was evaluated. In Treat 1 (CONTROL), cow [n = 130, 364.9 kg of body weight and 4.7 of BCS (1 to 9 scale)] received Ureado 50 P<sup>®</sup> (In-Vivo NSA, Paulínia, SP, Brazil). In Treat 2 (ENERGY) cows (n = 130, 364.1 kg of body weight and 4.8 of BCS) received energy supplement (MEGALAC-E<sup>®</sup>; Elanco, Nova Ponte, Brasil). The TAI was performed at 70 days postpartum. After TAI, cows were placed with bulls until the end of the 120 days of BS. Cows had their calves weaned at 110 days postpartum. b) Experiment 2 - The effect of the postpartum moment to perform the TAI on pregnancy rates of cows that had their calves weaned early was evaluated. In this experiment we used data from CONTROL group (TAI at 70 days postpartum) of the previous experiment and an additional group: MOMENT treatment cows [n = 65, weight = 369.7 kg, BCS = 4.8 (1-9)] received the same supplementation postpartum as Control, and TAI was performed 10 days after early weaning (at 110 days postpartum, the implant was placed on the day the calves were separated from their mothers). All cows were inseminated at fixed time with the protocol: D0: BE-2mg (RIC-BE<sup>®</sup>, Farmavet, Sorocaba, Brazil) and Primer<sup>®</sup> (Tecnopec-Agener União, São Paulo, Brazil); D8: device removal + d-cloprostenol-150µg (Prolise<sup>®</sup>, Arsa, Argentina) + 1mg of EB and D10: TAI using semen from the same batch of a single bull. Data were subjected to analysis using the GLIMMIX SAS version 9.12. The fixed variables were considered treatment, the category of differences were found, cow (primiparous or multiparous), inseminator and BCS were excluded from the model. In exp 1, similar (P>0.01) pregnancy per TAI (Control=38.5% and Energy=40.8%) and pregnancy rates at the end of BS was observed (Control=97.4% and Energy=98.3%). In Exp 2, also no difference between groups on pregnancy per TAI (Control=38.5% and Moment=41.5%) and on pregnancy rates at the end of BS was observed (Control=97.4 and Moment=91.5%). In conclusion, the postpartum moment to perform the TAI did not influence the pregnancy per AI of Nelore cows submitted to early weaning. Also, the energy supplementation by rumen-protected fat did not alter pregnancy per TAI and pregnancy rate at the end of BS in Nelore cows maintained in native pastures of the Pantanal.



A078 FTAI, FTET and AI

### Effects of exogenous progesterone on luteal function of high production dairy cows

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**Keywords:** corpus luteum, dairy cattle, progesterone.

We evaluated the effect of administration of injectable progesterone (P4; Sincrogest<sup>®</sup> injectable Ourofino, Brazil) on luteal function of lactating Holstein cows. A total of 83 cows, at a random stage of the estrous cycle, had their ovulation synchronized. Cows that ovulated after the synchronization (n=75; milk production of 32.5±0.6 kg/day, 95.8±4 days in milk, 1.5±1,0 lactations and body condition score of 2.9±0.6 - scale 1-5) were used in this study. On the third day of the estrous cycle, cows were allocated evenly according to the daily milk yield, days in milk, number of lactations, body condition score and follicle diameter at the time of ovulation into four experimental groups: Control (n=20) cows received no treatment; P4 (n=18) cows received 900mg im P4; P4+hCG (n=18) cows received 900mg P4 and 2000IU of hCG (Chorulon, MSD, Netherlands) im.; hCG (n=19) cows were treated with 2000IU i.m. of hCG. Blood samples were collected on D3, D5, D9, D13 and D17 of the estrous cycle to measure the plasma P4 concentration (ng/mL) and ultrasonographic examinations were performed on D5, D9 and D13 of the estrous cycle to measure the volume (mm<sup>3</sup>) of the corpus luteum (CL) excluding the cavities. Statistical analysis was performed using the SAS procedure GLIMMIX. The plasma P4 concentration among the control, P4, P4 + hCG and hCG groups were respectively; D3 (0.6±0.2; 0.7±0.06; 0.6± 0.05 and 0.5±0.08), D5 (1.5±0.1<sup>b</sup>; 2.4±0.2<sup>a</sup>; 3.0±0.3<sup>a</sup> and 1.2±0.1<sup>b</sup>), D9 (3.4±0.4<sup>b</sup>; 4.0± 0.4<sup>b</sup>; 5.9±0.9<sup>a</sup> and 4.2±0.4<sup>b</sup>), D13 (4.4±0.5; 4.5±0.5; 5.2±0.6 and 5.13±0.5) and D17 (4.12± 0.4; 3.3±0.5; 4.0±0.6 and 4.5±0.4). The CL volumes were respectively: D5 (2721±38; 2451±26; 3165±38 and 2645±39), D9 (6399±71<sup>b</sup>; 6661±69<sup>b</sup>; 8932±93<sup>a</sup>, and 8751±83<sup>a</sup>) and D13 (6007±62<sup>ab</sup>, 5417±36<sup>b</sup>, 7497±61<sup>ab</sup> and 7980±64<sup>a</sup>). There was an interaction between treatment and day of the estrous cycle (P=0.001) on plasma P4 concentration in P4 treated groups compared to the control group and hCG in D5 and D9. There was no interaction between treatments and day of the estrous cycle on CL volume, but there was a statistical difference (P=0.002) among groups treated with hCG compared to control and P4 in moments D9 and D13. We conclude that treatment with injectable P4 administration on D3 of the estrous cycle increases plasma P4 concentration from D5 to D9 of the estrous cycle without any influence on CL volume. The hCG treatment increased the volume of CL on D9 and D13 of the cycle.



A079 FTAI, FTET and AI

## Evaluation of ovulation time of suckled Nelore cows treated with eCG or calf removal in a nine day-based TAI protocol using different uses of CIDR

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**Keywords:** cow, reproduction, TAI.

The aim of this study was to evaluate different used CIDRs associated with different gonadotropic stimuli (GS) on follicular dynamic of suckled Nelore cows. A total of 117 cows received the following TAI protocol - D0: CIDR+2mg EB; D7: 12,5mg PGF; D9: Remove CIDR+0,5mg ECP. The cows were randomized to receive difference uses of CIDRs (1<sup>st</sup>: n=33; 2<sup>nd</sup>: n=33; 3<sup>rd</sup>: n=19 e 4<sup>th</sup>: n=32) and eCG at device removal (n=60) or calf removal (CR) during 48 hours from device removal to timed artificial insemination (n=57), 4x2 factorial arrangement. At the time of CIDR remove the cows had the tailhead painted with chalk marker, and had their estrus behavior monitored. Cows had their ovaries evaluated by ultrasound at Days 9 (0h), 11 (48h), 11.5 (56h) and 12 (72h), to follow the dominant follicle (DF) growth and to determine the moment of ovulation, and at Day 18 to detect the CL presence. The variables analyzed were DF 0h, DF 48h and follicular growth, FG), time of ovulation, P4 concentration and occurrence of estrus. The FG was calculated by the DF between 0 and 48h. Blood samples for P4 dosage were taken on 0h, 48h and Day 18. Data were analyzed using PROC MIXED of SAS. Significant effects were considered  $P \leq 0.05$ . There was no interaction between CIDR use and GS in analyzed variables ( $P > 0.1$ ). The follicle diameter at 0 and 48h and the difference between 48 and 0h were affect ( $P < 0.01$ ) by CIDR use: 1<sup>st</sup> use ( $8.2 \pm 0.29\text{mm}^a$ ;  $10.57 \pm 0.31\text{mm}^a$ ;  $2.25 \pm 0.23\text{mm}^a$ ), 2<sup>nd</sup> use ( $10.05 \pm 0.29\text{mm}^b$ ;  $12.07 \pm 0.31\text{mm}^b$ ;  $2.05 \pm 0.23\text{mm}^a$ ), 3<sup>rd</sup> use ( $10.63 \pm 0.38\text{mm}^b$ ;  $11.97 \pm 0.39\text{mm}^b$ ;  $1.33 \pm 0.28\text{mm}^b$ ), 4<sup>th</sup> use ( $10.84 \pm 0.30\text{mm}^b$ ;  $12.33 \pm 0.31\text{mm}^b$ ;  $1.41 \pm 0.23\text{mm}^b$ ); and no effect of GS in diameter of the DF at 0 and 48h, however tended to differ in FG ( $1.98 \pm 0.17\text{mm}$  vs.  $1.64 \pm 0.17\text{mm}$ ) for eCG and CR respectively. CIDR use affected P4 concentration ( $P < 0.01$ ) on day 9 (1<sup>st</sup>:  $5.06 \pm 0.24\text{ng/mL}^a$ , 2<sup>nd</sup>:  $3.80 \pm 0.24\text{ng/mL}^b$ , 3<sup>rd</sup>:  $3.02 \pm 0.30\text{ng/mL}^c$ , 4<sup>th</sup>:  $2.23 \pm 0.23\text{ng/mL}^d$ ), and there was no differences at 48h. Concentration of P4 on day 18 of ovulated cows only ( $P4 \geq 1\text{ng/ml}$  and presence of CL) was greater ( $P < 0.01$ ) on eCG treated ( $6.55 \pm 0.23\text{ng/mL}^a$ ) when compared to CR treatment ( $5.39 \pm 0.23\text{ng/mL}^b$ ). Cows that receive the 4<sup>th</sup> use CIDR had a higher proportion of estrus occurrence at 48h (87.5%; 28/32<sup>a</sup>) compared with 1<sup>st</sup> use (51.8%; 18/33<sup>b</sup>), 2<sup>nd</sup> use (60.6%; 21/33<sup>b</sup>) and 3<sup>rd</sup> use (53.3%; 11/19<sup>b</sup>). Cows treated with CR had higher proportion of estrus occurrence (39.2%; 22/57<sup>a</sup>) compared with eCG treated cows (16.5%; 11/60<sup>b</sup>). The proportion of cow that presented a ovulation with  $\leq 48\text{h}$  was 5.9% (7/117), between 48-60h was 15.3% (17/117), between 60-72h was 51.2% (60/117), and more than  $\geq 72\text{h}$  was 16.2% (19/117), and cows that did not ovulate was 11.1% (13/117). Greater proportion of cows synchronized with 4th use CIDR ovulated at 56h (40.6 %; 13/32<sup>a</sup>) when compared to the cows using CIDR 1<sup>st</sup> use (15.0%; 5/33<sup>b</sup>), 2<sup>nd</sup> use (15.2%; 5/33<sup>b</sup>) and or 3<sup>th</sup> use (11.1%; 2/19<sup>b</sup>). Higher proportion of cows treated with CR ovulating at 56hrs (28.9%; 17/57<sup>a</sup>) compared with eCG (12.0%; 8/60<sup>b</sup>). Synchronized cows with lower concentrations of P4 (i.e. 4th use of CIDR) and cows stimulated by CR had a higher proportion of premature ovulations after the CIDR removal.



A080 FTAI, FTET and AI

### **FTAI with injectable progesterone: ovarian follicular dynamics and pregnancy rates of Nelore (*Bos indicus*) cows, with and without corpus luteum**

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**Keywords:** follicular growth, injectable progesterone, ovulation.

The aim of this study was to evaluate the ovarian follicular dynamics and pregnancy rate of Nelore cows, with and without CL, synchronized for fixed-time AI (FTAI) with a parenteral injectable P4. Forty-four multiparous Nelore (72-84 months of age), 2.8±0.1 of BCS and 45 to 65 days postpartum were used. The experimental design was a 2x2 factorial, where 11 cows with and 11 without CL, received an intravaginal device with 1g P4 (DIB®, Syntex, Argentina) and 2mg EB (Syntex®, Syntex, Argentina) IM on D<sub>0</sub>. The device was removed on D<sub>8</sub> and animals were injected with 500µg cloprostenol (Cyclase®, Syntex, Argentina) and 300IU eCG (Novormon®, Syntex, Argentina) IM. On D<sub>9</sub> 1mg EB was given IM and FTAI performed 24h later. Eleven other animals with and 11 without CL received two applications of injectable P4 on D<sub>0</sub> (300mg<sup>1</sup> SC and 50mg<sup>2</sup> IM) and 2mg EB IM. On D<sub>6</sub> were applied 500µg cloprostenol and 300IU eCG IM. On D<sub>8</sub> 1mg EB was given IM and FTAI performed 24h later. The design of injectable protocol was performed according to previous studies (Unpublished data). Ultrasonography was performed on D<sub>0</sub>, D<sub>4</sub> to FTAI every 24h, from FTAI until ovulation every 12h and 12 days after ovulation. The presence of follicles ≥ 5 mm on D<sub>4</sub>, follicular growth rate, behavioral estrus rate, diameter of the dominant follicle (DF) at FTAI, ovulatory follicle (OF), ovulation rate, diameter of the CL and pregnancy rate were evaluated. The results were analyzed by ANOVA and Tukey test or by Chi-square test (P≤0.05). The presence of follicles ≥ 5mm on D<sub>4</sub> was similar between CL group - CLG (50% and 6.1±0.8mm) and group without CL - GwCL (55% and 6.6±0.8mm), and between the injectable group - IG (55% and 6.5±0.9mm) and the device group - DG (50% and 6.2±0.8mm). The follicular growth rate was similar between CLG (1.1±0.3mm/day) and GwCL (1.1±0.4mm/day), and between the IG (1.2±0.4mm/day) and the DG (1.1±0.3mm/day). The diameter of the DF at FTAI and OF were similar between CLG (10.5±1.8 and 11.4±1.3mm, respectively) and GwCL (10.8±2.3 and 11.1±2.2mm, respectively) but DG was higher than IG (11.7±2.0 and 11.8±1.7 vs. 9.7±1.7 and 10.6±2.0 mm, respectively; p<0.05). The CL size was similar between CLG (17.3±3.8mm) and GwCL (16.6±3.2mm), and between DG (17.6±3.5mm) and IG (16.1±3.4mm). The ovulation rate was similar between CLG (59%) and GwCL (77%) but higher in the DG (91%) than in the IG (45.5%; p<0.05). The pregnancy rate was similar between CLG (36%) and GwCL (27%) but higher in the DG (45%) compared to IG (18%; p<0.05). The CL present or absent did not affected the ovarian follicular dynamics and pregnancy rate. However, the synchronization of ovulation with intravaginal device showed higher diameter of the DF at FTAI, OF and ovulation and pregnancy rates compared to synchronization using P4 injectable.

<sup>1</sup>150mg/ml of natural P4 in base vehicle sesame and peanut oil (slow absorption).<sup>2</sup>50mg/ml of natural P4 in base vehicle sesame and peanut oil (fast absorption). \* Prepared in the laboratory only for studies.



A081 FTAI, FTET and AI

### **eCG influence the intracellular signaling pathways in bovine corpus luteum**

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**Keywords:** corpus luteum, eCG, follicular stimulation.

Using timed artificial insemination (TAI) techniques allied with equine chorionic gonadotropin (eCG) aims to increase the size of the dominant follicle and consequently the volume of the corpus luteum (CL), the progesterone (P4) production and conception rates. Changes in the regulation of the function and development of the CL in females stimulated with eCG were previously reported (Baruselli et al., 2011). This fact drove the hypothesis that treatment with eCG alters intracellular signaling pathways in the formed CL. To test the hypothesis 11 cows (*Bos indicus*) were divided into two groups: control and stimulated. Cows from both groups were treated with the same protocol for synchronization of follicular wave emergence, except for the administration of 400 IU of eCG (Novormon, Syntex, Buenos Aires, Argentina) on Day 8, which was only done in stimulated cows. At a random day of estrous cycle, animals all cows received an intravaginal device containing 1g of P4 (Primer, Technopec Brazil) and 2mg of estradiol benzoate (Estrogin, Farmavet, São Paulo, Brazil) intramuscularly. On Day 8, the intravaginal devices were removed, and 0.150 mg of d-cloprostenol (Prolise, Arsa, Buenos Aires, Argentina) was administrated. Forty eight hours after P4 devices removal, cows received 0.025 mg of Lecirelin (Gestran Plus, Arsa, Buenos Aires, Argentina). Six days after estimated ovulation (i.e. lecirelin treatment), all cows were slaughtered. Their CL were collected, weighed, measured, and frozen in liquid nitrogen. Partial results were collected from microarrays which were performed to identify differentially expressed genes between the two groups. The relative expression of genes involved in steroidogenesis signaling (*ADM*, *MMP9*, *NOS2*), activation of matrix metalloproteinases and protease activated receptor that regulates hemostasis and inflammation (*PRSS2*), angiogenesis (*ANG* and *ANGPT1*) and luteolysis (*PLAU*) were evaluated by qPCR. Decreased expression of genes related to the steroidogenesis (*MMP9*;  $P=0.01$ ) was found in the CL of eCG-treated cows, but expression of *ADM* did not change ( $P=0.41$ ) and the expression of *NOS2* was unaltered ( $P=0.87$ ), but was increased in stimulated group contrasting to the result of the microarray. On inflammation pathway gene expression, *PRSS2* ( $P=0.05$ ) was decreased. Regarding genes that interact in the angiogenesis pathway, the expression of *ANG* ( $P=0.02$ ) was increased in the CL of eCG-treated cows, while the expression of *ANGPT1* ( $P=0.11$ ) was similar in the CL of control and stimulated cows, like *PLAU* ( $P=0.41$ ) for luteolysis. In summary, the results showed herein are indicative that eCG changes the relative expression of genes that play important roles in the signaling pathways of the inflammation and angiogenesis. Thus, they may alter cellular responses related to the improvement of the CL function and, consequently, improve reproductive outcomes of cows submitted to TAI techniques.



A082 FTAI, FTET and AI

### Effect of different P4 devices over follicular dynamics of pubescent Holstein heifers submitted to timed artificial insemination

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**Keywords:** follicular dynamics, progesterone, TAI.

In heifers, high serum progesterone (P4) suppresses follicle growth by decreasing the LH pulsatile secretion. The aim of this study was to evaluate the effect of different P4 devices over follicular dynamics of pubescent Holstein heifers submitted to timed artificial insemination (TAI). A total of 20 pubescent Holstein heifers, in a random day of estrous cycle (D0) received an intravaginal devices containing 0.75 g of P<sub>4</sub> (*Prociclar*<sup>®</sup>, *HertapeCalier*, *Minas Gerais, Brazil*) in G1 (n = 10) and 1g of P<sub>4</sub> (*Primer*<sup>®</sup>, *Tecnopec*, *São Paulo, Brazil*) in G2 (n = 10) associated to 2mg of estradiol benzoate, im (*EB*, *Estrogin*<sup>®</sup>, *Farmavet*, *São Paulo, Brazil*). On D8, intravaginal P4 devices were removed, 500µg of cloprostenol were administered, im (*Ciosin*<sup>®</sup>, *Schering-Plough Animal Health*, *São Paulo, Brazil*), and 300 IU of eCG im (*Novormon*<sup>®</sup>, *Schering-Plough Animal Health*, *São Paulo, Brazil*). On D9, induction of ovulation was proceeded with 1mg BE, im (*Estrogin*<sup>®</sup>, *Farmavet*, *São Paulo, Brazil*) in both groups and TAI were done 54 hours after P4 device removal. The ultrasound examination was performed on D10, 4 hours after the TAI for measuring the diameter of the preovulatory follicle followed by examinations each 6 hours from D11 to time of ovulation. Data were analyzed using SPSS 16.0. There was no significant effect of treatment on these variables, the average diameter of the preovulatory follicle was 15.7 ± 0.45 mm. vs 14.2 ± 0.58 mm, for G1 and G2, respectively (P=0.08). Ovulation rate 70% vs 80% (P>0.05), mean time between removal of the intravaginal P4 device and ovulation 60.57 ± 3.17 hours vs. 70.0 ± 0.0 hours (P = 0.09) between G1 and G2, respectively. It can be verified that the concentration of P4 in the intravaginal devices with 0.75g and 1.0g did not influence the ovulatory follicle diameter nor the ovulation rate or the interval between the P4 devices removal and ovulation of pubescent Holstein heifers submitted to TAI.



A083 FTAI, FTET and AI

### Conception rates following FTAI of Nelore cows (*Bos indicus*) with high, intermediate and low numbers of antral follicles

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**Keywords:** antral follicle count, conception rate, FTAI.

The objective was to evaluate the influence of the population of antral follicles on the conception rate of *Bos indicus* cows with high, intermediate and low numbers of antral follicles during the follicular waves. Multiparous, suckling (40-60 days postpartum) Nelore cows (*Bos indicus*, n=701) with BCS  $3.0 \pm 0.5$  (1-5 scale) were submitted to a synchronization of ovulation protocol. Randomly during their estrous cycle (D0), they received an intravaginal progesterone (P4) device (CIDR®, Pfizer, São Paulo, Brazil) and 2 mg EB (Estrogin®, Farmavet, São Paulo, Brazil), i.m. At P4 device removal (D8), they were injected with 0.53 mg of cloprostenol (Ciosin®, Intervet-Schering Plough, Cotia, Brazil), 300 IU of eCG (Novormon®, Syntex SA, Buenos Aires, Argentina) and 1 mg of EC (ECP®, Pfizer, São Paulo, Brazil), i.m. Cows were timed inseminated 48h after P4 device removal. Antral follicles  $\geq 3$  mm were counted (D8) using an intravaginal microconvex array (Áquila PRO, Pie medical, Maastricht, The Netherland) and cows were assigned into groups with high antral follicular count (AFC; G-High,  $\geq 25$  follicles, n = 149), intermediate AFC (G-Intermediate, 11-24 follicles, n = 400) or low AFC (G-Low,  $\leq 10$  follicles, n = 152). Numbers of follicles were compared using the Kruskal-Wallis test and conception rates were compared using the Qui-square test, using a significance level of 0.05 (Bioestat 5.0). The overall mean number of antral follicles (mean  $\pm$  SD) was  $17.93 \pm 8.45$  and the overall conception rate was 51.49% (361/701). The mean population of antral follicles was  $30.70 \pm 5.66$  (G-High),  $17.03 \pm 3.28$  (G-Intermediate) and  $7.83 \pm 2.42$  follicles (G-Low,  $P < 0.05$ ). There was no difference on the conception rate among the groups of high and low AFC (51.67 versus 60.50%), however, conception rate of cows with low AFC was greater compared to the intermediate AFC group (60.50 versus 48.00%,  $P < 0.05$ ). Thus, it is concluded that Nelore cows with low AFC had greater conception rate following FTAI protocol compared to cows with intermediate AFC.



A084 FTAI, FTET and AI

### **Pregnancy rates following FTAI of Nelore heifers (*Bos indicus*) with high, intermediate and low numbers of antral follicles**

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**Keywords:** antral follicle count, *Bos indicus*, FTAI.

The objective of this study was to evaluate the influence of the population of antral follicles on the conception rate of Nelore heifers submitted to fixed-time AI (FTAI). Two hundred and eight pubertal Nelore heifers with body condition score (BCS) of  $3.0 \pm 0.5$  were submitted to a synchronization of ovulation protocol. On a random day of the estrous cycle (D0), heifers received an ear implant containing norgestomet (Crestar<sup>®</sup>, Intervet Schering-Plough, Brazil), 2mg BE (Estrogen<sup>®</sup>, Farmavet, Brazil) and 1 mg cloprostenol (Ciosin<sup>®</sup>, Intervet Schering-Plough, Brazil), IM. At implant removal (D8), heifers received 1mg of cloprostenol (Ciosin<sup>®</sup>, Intervet Schering-Plough, Brazil), 300IU eCG (Novormon<sup>®</sup>, Syntex SA, Argentina) and 0.5 mg of estradiol cypionate (ECP<sup>®</sup>, Pfizer, Brazil) IM. Heifers were timed inseminated 48 hours after the implant removal. Antral follicles  $\geq 3$  mm were counted by using ultrasound transducer microconvex of 5 MHz and heifers were divided into groups according to the antral follicle count (G-High,  $\geq 30$  follicles, n = 38, G-Intermediate, 13-29 follicles, n = 143, G-Low,  $\leq 12$  follicles, n = 27). Pregnancy diagnosis was performed by transrectal ultrasonography (5 MHz), 30 days after FTAI. The number of follicles was evaluated by Kruskal-Wallis and pregnancy per AI (P/AI) were compared by Chi-square test ( $P \leq 0.05$ ). There was no interaction between BCS and AFC. The overall average number of antral follicles (mean  $\pm$  SD) was  $21.48 \pm 9.47$  and the overall P/AI was 44.71% (93/208). The average of follicular population observed in groups G-High, G-Intermediate, and G-Low was  $37.73 \pm 7.05$ ,  $19.23 \pm 4.29$  and  $10.55 \pm 2.17$  follicles, respectively. There was no difference on P/AI among groups (G-High: 44.73%, G-Intermediate: 43.35%, G-Low: 51.85%,  $P > 0.05$ ). The present data suggest that the variations in the population of antral follicles do not influence P/AI of heifers submitted to FTAI.



A085 FTAI, FTET and AI

### **Circulating progesterone concentrations according to ovarian luteal activity of heifers treated with intravaginal devices**

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**Keywords:** FTAI, progesterone, reproduction.

Progesterone (P4) is an important hormone in fixed-time artificial insemination (FTAI) programs (Costa et al. 1999, Braz J AnimSci, 28, p.1243-1247). Hormonal protocols used in FTAI programs involve considerable financial values, in which P4 represents 43% of the total cost. The P4 releasing from intravaginal devices occurs by passive diffusion (Brunton et al. 2008, Manual of Pharmacology and Therapeutics, 4<sup>th</sup> Ed., 1254p), i.e., the hormone release is driven by a concentration gradient and enhanced by the contact area between the device and vaginal epithelium (Rothen-Weinhold et al. 2000, J Dairy Sci, 83, p.2771–2778). Given the importance of P4 in the protocols (Savio et al. 1993, J. Reprod.Fertil. v. 98, p. 77-84) and the representativeness of this steroid in the synchronization treatment costs, several studies described the reutilization of P4 devices as an alternative to make this technology viable (Almeida et al. 2006, Braz. J. Vet. Res. Anim. Sci., 43, p. 456-465). However, the results are controversial and the P4 releasing pattern from intravaginal devices in animals with different luteal activity (amount of endogenous P4 synthesis) is not described. The aim of the present study was to evaluate the P4 profile in heifers with different ovarian luteal activity treated with a new intravaginal device (1.0 g of P4) for 8 days. Animals were allocated into 3 groups: Group 1 – with corpus luteum (CL) during all treatment period; Group 2 – with CL during half of treatment period; Group 3 – without CL. At the beginning of the treatment (D0), the animals from G1 and G2 had a functional CL formed 8 days before implant insertion. In G2 animals, 150µg of D-cloprostenol (Veteglan, Hertape Calier, Brazil) was administrated 3 days after implant insertion (D3). Animals from G3 did not have luteal function (i.e. absence of CL) at the beginning of the treatment. Blood samples were collected on D0 (moment of intravaginal P4 device insertion), D3, D5, and D8. Serum P4 concentration was determined by radioimmunoassay. Mean plasma P4 concentration on each day was compared by Tukey's test. Serum P4 concentration in the groups G1 and G2 was greater than in G3 on D0 (5.34<sup>a</sup>; 5.30<sup>a</sup> and 0.64<sup>b</sup> ng/mL, respectively; P<0.05) and also on D3 (5.76<sup>a</sup>; 5.46<sup>a</sup> and 3.61<sup>b</sup> ng/mL, respectively; P<0.05). On D5, 36 hours after D-cloprostenol treatment in G2, serum P4 concentration of those heifers was similar to G3, and both were lower than G1 (3.36<sup>a</sup>, 2.46<sup>b</sup> and 2.15<sup>b</sup> ng/mL, for G1, G2, and G3 respectively; P<0.05). Similar result was observed on D8 (3.19<sup>a</sup>, 1.84<sup>b</sup> and 1.58<sup>b</sup> ng/mL for G1, G2, and G3 respectively; P<0.05). Moreover, difference of the serum P4 concentration between D3 and D0 in G1 and G2 were different when compared to G3 (0.46<sup>a</sup>; 0.19<sup>a</sup> and 2.83<sup>b</sup> ng/mL, respectively; P<0.05). In conclusion, the present results suggest that P4 release from intravaginal device is directly influenced by blood P4 concentration of the treated animal. Thus, animals with a functional CL during FTAI protocol may consume less P4 from the intravaginal P4 device when compared to those without CL.



A086 FTAI, FTET and AI

## **Effects of the components of artificial insemination technique on fertility of white Leghorn hens**

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**Keywords:** artificial insemination, fertility, white leghorn chickens.

Artificial insemination (AI) of chickens in cages has been shown to be technically feasible. However some of the technical procedures could contribute to the variation in the fertility results. To ascertain the effects of some components factors of the technical protocols of AI, in poultry fertility rates, were comparatively evaluated data in the file of Pesagro-Rio. We used the records of 480 White Leghorn chickens (two per cage) and 50 roosters selected with routine procedures for semen collection and AI performed as described in the artificial insemination manual of hens of the PESAGRO-RIO (Resende et al. 1983 PESAGRO-RIO, Technical Bulletin, n. 6. 16p.). The design was a randomized block in a factorial of two levels of factors of the number of AI by week (two and one); semen concentration (undiluted and diluted 1:1 in Locke-Ringer solution), dose volume (0.05 and 0.025 mL), semen preservation temperature (37 to 15°C) and in blocks periods during the process of AI (0-15, 15-30 and 30-45 minutes). The data were subjected to analysis of variance and t test in the Assistat Program (Silva, F. A. S., 2013. Assistat-Statistical Assistance Software – UFCG, Paraíba, Brasil. <http://www.assistat.com>). The evaluation results showed that the fertility rates in relation of the levels of isolated factors were, respectively, 96.1 and 89.1% for two and one AI by week ( $P < 0.01$ ); 93.2 and 91.8% for the undiluted and diluted semen ( $P > 0.05$ ); 92.0 and 93.1% for doses of 0.05 and 0.025 mL ( $P > 0.05$ ); 91.5 and 93.3% for storage temperatures of 37 and 15°C ( $P > 0.05$ ); 93.6, 91.6 and 92.3% for the preservation times of semen in periods of 0-15, 15-30 and 30-45 minutes ( $P > 0.05$ ). Interactions between treatments were not significant. Fertility rates were high, and only the number factor AI per week showed significant effect on fertility outcomes with the reproductive technique of AI in White Leghorn chickens.



A087 FTAI, FTET and AI

## Effect of the number of daily observation on heat detection efficiency in dairy herds

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**Keywords:** dairy herd, detection efficiency, heat.

The aim of this trial was to compare the impact of the number of daily observation (2 vs 6 observation/day) on heat detection efficiency in Holstein dairy herds. The trial was conducted in three commercial herds located in Cuenca Mar and Sierras region in the south east of Buenos Aires province, Argentina. Herds 1, 2 and 3 had 74, 143 and 201 lactating cows, respectively. The average ( $\pm$ SE) production 305 days, DIM and number of lactation were: 7,536 $\pm$ 1,565.2, 132.9 $\pm$ 53.3 and 2.5 $\pm$ 1.5 for herd 1; 7364 $\pm$ 1242.3, 155.8 $\pm$ 100.9 and 4.0 $\pm$ 1.5 for herd 2; and 7,767.8 $\pm$ 1,313.8, 89.5 $\pm$ 46.2 and 2.3 $\pm$ 1.7 for herd 3. The observations were done at 4:00 am; 08:00 am; 11:00 am; 03:00 pm; 06:00 pm and 09:00 pm during 45 min each. For the 2 observation group, only cows in heat at 8:00 am and 06:00 pm were considered. Tail paint was used as an auxiliary method for heat detection. The cow was considered in heat when standing heat was detected as well as if more than 50% of the tail paint has disappeared. Statistic evaluation was made evaluating proportion using the Test of Mac Nemar. Heat detection efficiency was increased when cows were observed 6 times daily (69.6%; 291/418) compared to two times daily (27.0%; 113/418: P=0.001). Considering each herd, heat detection efficiency for cows observed 2 and 6 times was respectively: 33.8% (25/74) and 75.7% (56/74) for herd 1 (P<0.001); 25.9% (37/143) and 50.3% (72/143) for herd 2 (P<0.001); and 25.4% (51/201) and 81.1% (163/201) for herd 3 (P<0.001). In conclusion 6 daily observation increases heat detection efficiency in dairy herds. This management can allow increased number of eligible cows to be inseminated (i.e. greater service rate) and thus improve reproductive efficiency in dairy herds.



A088 FTAI, FTET and AI

### Supplementation with organic selenium source on the fertility of Nelore cows

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**Keywords:** FTAI, minerals, supplementation.

Selenium has been shown to be very important for reproduction. It has higher bioavailability in organic form. The aim of this study was to evaluate the influence of organic source of selenium composed by antioxidant, replacing the inorganic source of selenium on pregnancy rate Nelore cows. The experiment was realized in the Santa Marta farm, Campo Florido, MG. Nelore cows (n=338) were allocated into two groups: 1) Control- supplementation with inorganic source of selenium (Premiphos Monta<sup>®</sup>, Premix) and 2) Treatment- supplementation with organic source of selenium (EconamasE<sup>®</sup>, Alltech), both supplemented during all 90-days of the breeding season (BS). On day 50 of the BS a timed artificial insemination (TAI) was performed. Follicle diameter (ØDF) on D9 (i.e. one day after the intravaginal progesterone [P4] removal) of the TAI program, and corpus luteum diameter (ØCL), 7 days after TAI were measured by ultrasonography (DP-2200 Vet<sup>®</sup>, Mindray, China). Blood samples were collected for P4 concentration and lipid peroxidation (TBARS) analysis 7 days after TAI. Mean body weight before the beginning of supplementation, on day 50 (moment of TAI) and at the end of 90-d of the BS were evaluated. The TAI program consisted of D0: 2 mg estradiol benzoate (EB, RIC-BE<sup>®</sup>, Tecnopec-Agener União) plus an intravaginal P4 device (Primer<sup>®</sup>, Tecnopec-Agener União); D8: P4 device withdrawal, 150 µg *d*-cloprostenol (Prolise<sup>®</sup>, Arsa, Argentina), 10 mg FSH<sub>p</sub> (Folltropin-V<sup>®</sup>, Bioniche, Canada) and 1 mg EB. On D10, cows were timed inseminated and pregnancy diagnosis was performed 45 d after TAI. The statistical analysis was accomplished by SAS program, using the variance analysis by Tukey and Chi-square test (P<0.05). Pregnancy per TAI and ØDF were similar between groups, respectively [Control = 42.2% (71/168) and 11.2±2.4 mm vs Treatment = 46.5% (79/170) and 10.9±2.1 mm]. However, ØCL (16.5±3.1 vs. 18.2±2.5 mm), P4 concentration (2.1±1.3 vs. 2.5±1.5 ng/mL), mean body weight (427.3±59 vs. 445.0±65 kg) and TBARS (0.097 vs. 0.039 n/mols) were different between groups (control vs. treatment, respectively). Organic mineral supplementation improved CL diameter, P4 concentration and lipid peroxidation levels; however, did not influence the pregnancy per TAI in Nelore cows.

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A089 FTAI, FTET and AI

## Increasing the interval between luteolysis and AI decreases pregnancy loss in lactating dairy cows submitted to E2/P4 TAI program

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**Keywords:** estrus, luteolysis, pregnancy loss.

The hypothesis of this study was that increasing interval between luteolysis and artificial insemination (AI) would improve fertility to the timed AI (TAI) program. Lactating Holstein cows (n=759) yielding 30.5±8kg of milk/d, with a detectable CL at US (D-11) were randomly assigned to receive either one of the following treatments: 1) 3d interval between luteolysis and TAI: D-10 = CIDR insertion (CIDR®, 1.9g P4, Zoetis, São Paulo, Brazil) plus 2.0 mg im of estradiol benzoate (EB; Estrogen®, Farmavet, São Paulo, Brazil); D-3=PGF<sub>2</sub>α im (25mg dinoprost tromethamine, Lutalyse®, Zoetis, São Paulo, Brazil); D-2 = CIDR removal plus 1.0 mg of ECP im (ECP®, Zoetis, São Paulo, Brazil); D0 = TAI; 2) 4d interval between luteolysis and TAI: D-11 = CIDR insertion plus 2.0 mg of EB im; D-4 = 25 mg of PGF<sub>2</sub>α im; D-2 CIDR removal plus 1.0 mg of ECP im; D0 = TAI. Cows were considered synchronized following to the protocol when a CL was detected on D7 (i.e. 7 days after TAI). Pregnancy diagnoses were performed on D32 and D60. Data were analyzed by PROC GLIMMIX of SAS. Significance level was defined as  $P<0.05$ . The largest follicle diameter at TAI did not differ ( $P=0.30$ ) between treatments (3d=14.7±0.39 x 4d=15.0±0.40mm). Synchronized cows treated with the 4d program tended ( $P=0.06$ ) to have higher P4 concentrations at D7 (3.14±0.18ng/mL) than synchronized cows treated with the 3d program (3.05±0.18ng/mL). Although the P/AI at D32 [3d=45%(175/385) vs. 4d=43.9%(166/377)] and at D60 [3d=38.1%(150/385) vs. 4d=40.0%(154/377)] were not different between groups, the 4d program determined lower ( $P=0.04$ ) pregnancy loss (7.6%;12/166) than the 3d program (14.7%;25/175). The P/AI at 60d was reduced ( $P<0.01$ ) in cows that ovulated a smaller follicle (<11mm=37.2%; 22/66) or a larger follicle (>17mm=29.3%; 39/128), compared to cows that ovulated follicles between 11 and 17mm (49.1%; 197/395). The 4d program-treated cows were more likely ( $P<0.01$ ) to display estrus (73.0%; 269/374) than 3d program-treated cows (63.4%; 240/385). The occurrence of estrus improved ( $P<0.01$ ) the synchronization (97.4%; 489/501 vs. 81%; 202/248), P4 concentrations at D7 (3.22±0.16 vs. 2.77±0.17ng/mL), P/AI at D32 (51.2%; 252/489 vs. 39.4%; 81/202), P/AI at D60 (46.3%; 230/489 vs. 31.1%; 66/202); and reduced ( $P<0.01$ ) the pregnancy loss (9.3%; 22/252 vs. 19.8%; 15/51), compared to cows that did not display estrus, respectively. Cows that did not display estrus within small (<11mm) or big follicles (>17mm) at TAI had higher pregnancy loss ( $P=0.01$ ). However, in those cows that displayed estrus, the follicle diameter at TAI did not affect ( $P=0.97$ ) the pregnancy loss. In conclusion, increasing the interval between luteolysis and TAI increased the estrus occurrence and reduced the pregnancy loss of lactating dairy cows submitted to E2/P4 TAI program.



A090 FTAI, FTET and AI

### Progesterone treatment during the periovulatory period decreases embryo production in superovulated buffaloes

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**Keywords:** buffaloes, embryos, Superovulation.

The present study evaluated the hypothesis that elevated progesterone (P4) during the expected multiple ovulations can improve the embryo recovery rate in superovulated buffalo donors due to decreased estradiol concentrations and contractility of genital tract. Buffalo donors cows were homogeneously assigned into 2 groups, according to age, body condition score, days postpartum and ovarian activity (presence of CL), in a cross over experimental design (35 d interval between superovulations): Control (C-G; n=8) and P4 treatment during the periovulatory period (P4-G; n=8). The follicular wave emergence was synchronized with an intravaginal P4 device (Sincrogest<sup>®</sup>, Ourofino Agronegócio, São Paulo, Brazil) plus 2 mg i.m. of estradiol benzoate (Sincrodiol<sup>®</sup>, Ourofino Agronegócio, Brazil) administrated at a random stage of the estrous cycle (Day 0=D0 AM). All buffaloes received 200 mg i.m. of FSH (Foltropin-V<sup>®</sup>, Bioniche Animal Health, Canada) twice-daily, in 8 decreasing doses starting on D4 AM. A dose of PGF<sub>2α</sub> (530µg i.m. Sincrocio<sup>®</sup>, Ourofino Agronegócio, Brazil) was given on D6 PM and on D7 AM. The P4 device was removed from cows of the C-G on D7 PM and from cow of the P4-G on D10 PM. On D8 PM, all buffaloes received 25 mg i.m. of pLH (Lutropin-V<sup>®</sup>, Bioniche Animal Health). The timed inseminations were done 12 and 24 h after the pLH treatment. Blood samples were collected from D7 (PM) to D11 (AM) for further progesterone assay. The structures (ova/embryos) were collected nonsurgically 6 days after the second TAI (D14 PM). Transrectal ovarian ultrasound examinations (Mindray, DP2200Vet, China) were performed on D0 to verify ovarian activity, on D8 PM and on D14 to verify respectively, the superstimulation and the superovulatory responses. The variables were analyzed by GLIMMIX procedure of Statistical Analysis System (SAS<sup>®</sup>). On D8, was verified similar number of follicles >8mm in both groups (P4-G=12.1±3.2 vs. C-G=11.0±2.7; P=0.68). Buffaloes from P4-G group showed lower ovulation rate (13.5±4.9 vs. 71.5±16.1 %; P=0.002) and lower number of CLs on D14 (1.1±0.3 vs. 8.0±2.8; P=0.04) than buffaloes from C-G group, respectively. The number of recovered structures, transferable and freezable embryos to C-G and P4-G were: 0.0±0.0 and 1.9±0.7 (P=0.03); 0.0±0.0 and 1.6±0.7 (P=0.04); 0.0±0.0 and 1.6±0.7 (P=0.04). The serum progesterone concentration measured from D7 to D11 was greater in the P4-G (1.87±0.13) than in the C-G (0.48±0.10, P <0.0001). It is possible that the high serum progesterone concentration due to the maintenance of the intravaginal P4 device during the periovulatory period was responsible for reducing the ovulation and the absence of embryonic structures in P4-G, rejecting the present hypothesis.



A091 FTAI, FTET and AI

### Cooled semen for fixed-time artificial insemination in cattle

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**Keywords:** *Bos indicus*, dilution, synchronization.

Cryopreservation process causes damage in the cells that affects the viability and may compromise the pregnancy per artificial insemination (P/AI) and reproductive efficiency. Thus, the aim of this study was to evaluate the possible use of cooled semen in fixed-time artificial insemination (FTAI) program compared with frozen semen to improve P/AI in beef cattle. Ejaculates of three bulls were collected and divided into two treatments: 1) frozen semen; and 2) chilled semen, maintained at 5°C, for 24 h. All proceedings were the same until the cooling process. Egg-yolk extender without glycerol was used for the cooling process. Straws of 0.25 mL with 25x10<sup>6</sup> sperm were submitted to the cooling process in a cooled room for the cryopreservation process (-196°C); or in a Botu-Tainer<sup>®</sup> box for preservation at 5°C until 24 h later, when FTAI was performed. The straws were thawed at 35°C, for 20 sec. Chilled semen did not undergo the thawing process and it was used immediately after being removed from the box. Semen samples were evaluated after collection, dilution, post-thaw and cooling process for motility, thermoresistance and hyposmotic test, by the same operator. Postpartum multiparous Nelore cows (n=838) were submitted to FTAI with the following protocol: D0: estradiol benzoate (EB, 2mg, RIC-BE<sup>®</sup>, im, Tecnopec-Agener União) and a progesterone device (Primer<sup>®</sup>, Tecnopec-Agener União); D8: device withdrawal, *d*-cloprostenol (PGF2 $\alpha$ , 150 $\mu$ g, Prolise<sup>®</sup>, Arsa, Argentina), 1mg EB plus 300 IU eCG (Novormom<sup>®</sup>, Syntax, Argentina) im administrations; and D10: AI using frozen semen (n=408) or cooled semen (n=430). The statistical analysis was accomplished using the GLIMMIX procedure of SAS for pregnancy rate and with ANOVA followed by Tukey for complementary tests (P<0.05). There was ~20% increase in pregnancy rate with the use of cooled semen compared with frozen semen (59.9% vs. 49.4%; P<0.05). There was no difference among bulls for pregnancy (P=0.39). Pregnancy rates for bull semen frozen and cooled were, respectively, 45.8% vs 55.4% (bull 1, n=330), 56.4% vs 76.4% (bull 2, n=114) and 51% vs 56.2% (bull 3, n=394). Frozen semen had less viable sperm than cooled semen evaluated by sperm motility (61.7% $\pm$ 7.6 vs 81.0% $\pm$ 10.4), thermoresistance (41.7% $\pm$ 7.4 vs 66.7% $\pm$ 10.4) and hyposmotic (38.3% $\pm$ 7.6 vs 53.7% $\pm$ 9.9) tests. The use of cooled semen from bulls genetically evaluated in breeding programs, compared with frozen semen, increased the P/AI in multiparous postpartum Nelore cows submitted to a FTAI protocol. This is an interesting alternative to increase the reproductive outcomes and reduce the costs of the FTAI program.

**Acknowledgments:** CNPq, Agener União Saúde Animal, Ema Pantanal Ltda and Melhor Animal Ltda.



A092 FTAI, FTET and AI

## The economic impact of the use of TAI or natural service to rebreed cows submitted to TAI programs on cow-calf operation

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**Keywords:** costs, Nelore, pregnancy.

The use of timed artificial insemination (TAI), associated with natural service for rebreed non-pregnant cows, adds the costs of the synchronization for TAI program with the necessity of maintenance of bulls, that could reduce the profitability of cow-calf production system. At the same time, there is a wide variety of hormonal protocols and cost of semen dose, which represent an alternative to improve the cost-effectiveness of the reproductive program. This experiment aimed to evaluate the cost-benefit ratio when using only TAI (three inseminations) and TAI (two inseminations) associated with the rebreeding using bulls. Herein was evaluated the pregnancy outcomes obtained and the cost per pregnancy produced in each system. One hundred cows and one hundred Nelore heifers were randomly divided into four groups: G1 - Heifers + three TAI; G2 - Heifers two TAI + rebreeding using bulls (NS); G3 - Cows + three TAI; G4 - Cows two TAI + NS. At a random day of the estrous cycle (D0), the animals received an ear implant containing 3 mg of norgestomet (Crestar<sup>®</sup>, MSD Animal Health, São Paulo, Brazil) and intramuscular injection of 2 mg of estradiol benzoate (Gonadiol<sup>®</sup>, IM, MSD Animal Health, Brazil). The ear implant was removed on D8, along with intramuscular administration of 300 IU of equine chorionic gonadotropin (Novormon<sup>®</sup>, MSD Animal Health, São Paulo, Brazil), 1 mg of estradiol cypionate (E.C.P<sup>®</sup>, IM, Zoetis, São Paulo, Brazil) and 350 mg Sodium cloprostenol (Ciosin<sup>®</sup>, MSD Animal Health, São Paulo, Brazil). The animals were timed inseminated on D10 and the pregnancy diagnosis was performed 28 days after each TAI. Non-pregnant females were submitted to a new synchronization protocol. The groups 1 and 3 were submitted to three TAI synchronization protocols. Groups 2 and 4 underwent two TAI synchronization protocols and eight days after the second TAI, females were placed together with bulls for adding 45 days. Twenty-eight days after the removal of the bulls the pregnancy diagnosis was performed by ultrasound (Mindray Vet-2200) in all breeding groups. Data were analyzed by ANOVA using F test (SISVAR). There was no difference (Tukey, P<0.10) in overall pregnancy rates obtained: 78.85% with production cost of R\$ 66.91 per pregnancy in G1 group, 87.96% of pregnancy rate with cost per pregnancy of R\$ 76.51 in the G2 group, 85.42% of pregnancy rate with a cost of R \$ 56.36 in the G3 group, and 90.57% pregnancy rates with a cost of R\$ 81.47 in the G4 group. In addition, the costs for production of calves did not differ among treatments. Considering the value of marketing on average R\$ 700.00 for calves weaned at eight months old, the costs represent 9.5% of the calves produced for G1, 10% for G2, 8% for G3 and 12% for group 4. Therefore, it is possible to achieve good reproductive rates at the end of the breeding season with satisfactory cost-benefit ratio using only three TAI programs without the use of bull service.



A093 FTAI, FTET and AI

### Which is the best protocol for cervical dilatation in sheep embryo collection?

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**Keywords:** ketamine, misoprostol, oxytocin.

The development of methodologies for the transcervical embryo collection in sheep is strongly encouraged by the sequelae caused by conventional surgical methodology. However, this technique is impaired by the fact that, on average, only 30-40% of the animals have a pervious cervix. Three transcervical approaches in ewes are described: prostaglandin E (PGE; Gusmão et al., 2007, Rev. Bras. Saúde Prod. An. 8, 1-10), estradiol+oxytocin (EO; Masoudi et al., 2012, Afr. J. Biotech. 11, 2803-2806) or subarachnoid anesthesia with ketamine (AK; DeRossi et al., 2009, Small Rumin. Res. 83, 74-78). However, there is no comparative information on what is the most efficient. Thus, this study was designed in order to compare the cervical dilator efficiency of treatment with PGE analogue (Misoprostol; Prostokos<sup>®</sup>, Hebron, BR), EO (Estradiol benzoate; Estrogen<sup>®</sup>, Farmavet, BR + Synthetic oxytocin; Placentex<sup>®</sup>, Agener União, BR) or AK (Ketamine; Dopalen<sup>®</sup>, Vetbrands, BR) in Crioula Lanada ewes. Initially, the cervical transposition of the animals (n=18) was evaluated under physiological dilation (estrus) using a Hegar's dilator № 2 in a maximum period of 7min. The cervixes were classified as fully pervious (FP,  $\geq 6$  cm), partially pervious (PP, from 4 to 5.9 cm) and non-pervious (NP,  $\leq 3.9$  cm) for homogeneous distribution of animals (n=6) across treatment groups in order to evaluate the effect of cervical dilator treatments at diestrus (Day 6). On Day 5, 12h before the evaluation, animals from PGE and EO groups received one intravaginal tablet of misoprostol (200 $\mu$ g) and an IM injection of estradiol benzoate (100 $\mu$ g), respectively. On the morning of Day 6, the animals from PGE group were immediately evaluated and those from EO and AK groups were evaluated 15min after an IM application of oxytocin (100 IU) and a subarachnoid injection of ketamine (1.5mg/kg), respectively. The evaluation during estrus determined that 44.4%, 22.2% and 33.3% of the animals had FP, PP and NP cervixes, respectively. During diestrus, in PGE group one animal maintained FP classification and another went from PP to FP while the others were NP. In this group it was observed that most of the intravaginal tablet was expelled by the animals, which probably impaired the effect on cervical dilatation. In the EO group, one of two FP animals went to PP and from two initially PP one had increased in the depth and the other became FP. From two NP animals one became PP and the other FP, resulting in 83% dilatation. In AK group, from three classified as FP, one became NP and the PP animal became NP. In this group, beyond the low cervical dilator response, one animal had complications from an unexpected reaction during insertion of the needle into the subarachnoid space. In conclusion, due to the increased possibility of cervical transposition, the treatment with a combination of estradiol benzoate + oxytocin is the most suitable for transcervical embryo collection in sheep.



A094 FTAI, FTET and AI

## Ovulation and pregnancy rates in Nelore cows submitted to timed AI

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**Keywords:** FTAI, Nelore, ovulation.

The number of cows inseminated by the fixed-timed artificial insemination (TAI) in Brazil has been largely increased due to the simplification of the necessary management and the possibility of insemination with no needs of estrus detection, especially in large scale beef herds. Several pharmacological protocols for the control of the estrus and ovulation of cattle have been proposed to improve the TAI efficiency and facilitate the reproductive management. An important change in the traditional protocol called PEPE (progesterone [P4]-estradiol-prostaglandin-estradiol) is using estradiol cypionate on the same time of implant removal, reducing the management with the animals. The aim of this field study was to compare two TAI protocols using estradiol cypionate or estradiol benzoate (EB) compared with the use of PEPE synchronization protocol during the 2012/2013 breeding season. Also, it was evaluated the possibility to perform the AI after the detection of estrus signs on the day of the beginning of the protocol (D0). Multiparous Nelore cows (n=96) of a commercial herd, with more than 45 days postpartum, were assigned into one of two homogeneous groups and subjected to different TAI protocols. The two management protocols had P4 device insertion (Sincrogest, Ourofino Animal Health, São Paulo, Brazil) and 2.0mg im of EB (Gonadiol®; MSD, São Paulo, Brazil) on a random day of the estrous cycle (D0). Eight days later, the P4 device was removed and 1.0mg of EB and 12.5 mg of dinoprost (Lutalyse®, Pfizer, São Paulo, Brazil) were administered im. The TAI was performed 54 to 56 hours (D10) after the device removal. In the PEPE protocol, the P4 device insertion and 2.0mg EB was administered on D0. On D7, the P4 device was removed and dinoprost was administered. Twenty four hours later, 1.0 mg of EB was administered and the TAI 34 to 36 hours after the last EB treatment (D9). All animals underwent a gynecological examination by transrectal ultrasonography (US) to confirm the presence of a dominant follicle (DF) at the time of TAI. Pregnancy diagnosis was performed 30 days after TAI by US. In the two management protocols, 10.41% (5/48) of the cows became pregnant and 30% (12/40) showed FD at the TAI. However, in the PEPE protocol, 43.75% (21/48) of the cows became pregnant and 91.89% (34/37) had FD at the TAI moment. In the beginning of the protocols, 20 cows showed estrus signs and were inseminated 10 to 12 h after (D0), but none became pregnant. A simple observation of estrus signs in the first day of the protocol is not sufficient to carry out the AI. Changes in pharmacological synchronization protocols should be carefully considered before its indication for commercial use.

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A095 FTAI, FTET and AI

### Follicular dynamic of Holstein cows superstimulated with FSH associated with a slow release diluent for *in vivo* embryo production programs

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**Keywords:** donors, non lactating cows, superovulation.

The aim of the present study was to evaluate the follicular dynamic of Holstein cows submitted to superstimulation protocols with FSH (Folltropin, Agener Union, Brazil) associated with a diluent with slow release carrier (hyaluronic acid; MAP5, Bioniche). The cows (non-lactating) were randomly allocated into one of two treatments: Folltropin (FOLL, n=8) or Folltropin diluted in MAP5 (MAP5, n=8). In random day of the estrous cycle (D0) females received an intravaginal P4 device (Primer, Tecnopec) and 2.0 mg IM of EB (RIC-BE, Tecnopec). From D4 on, FOLL group received 300 mg of Folltropin diluted in 20 mL of saline and administrated in 8 decreasing doses, with an interval of 12h. The MAP5 group received the same dose of Folltropin diluted with MAP5 and fractionated into two applications in decreasing doses (5.0 and 2.5ml) on day D4 and D6. On D6 (am and pm) 0,150 mg of cloprostenol (Estron, Tecnopec) were administered. Still, P4 implants were removed on D7 pm and 12h later 62.5µg IM of Lecirelin (Gestran, Tecnopec) were administered. Ultrasonography examinations were performed on D4, D6 and D8 to quantify and measure the ovarian follicles. Follicles were classified as small (SF; Ø<8mm), medium (MF Ø≥ 8 and <10mm) and large (LF; Ø≥10mm). Still, on D15 the number of CL was recorded to evaluate the ovulation rate (number of CL on D15 per number of LF). Statistical analysis was performed using the GLIMMIX procedure of SAS. Data are presented as mean±SEM or percentage. Values with superscript letters within treatment (<sup>A≠B≠C</sup>) and within time (<sup>x≠y</sup>) differ. In both treatments there was a reduction in the number of SF over time, however, the decrease was more accentuated in the FOLL group (MAP5- D4:16.1±2.7<sup>Ax</sup>, D6:11.3±2.7<sup>Bx</sup> and D8:6.3±3.0<sup>Cx</sup>; FOLL-D4:18.4±4.0<sup>Ax</sup>, D6:6.1±1.7<sup>By</sup> and D8:2.1±0.9<sup>Cy</sup>; P<sub>TREAT</sub>=0.002, P<sub>TIME</sub><0.0001 and P<sub>TREAT\*TIME</sub>=0.0002). Regarding the number of MF, unlike MAP5 group (D4:0.9±0.4<sup>Bx</sup>, D6:2.4±0.9<sup>Ax</sup> and D8:2.1±0.9<sup>ABx</sup>), FOLL group had an increased on D6 (9.7±3.2<sup>Ay</sup>) compared to D4 (1.1±0.4<sup>Bx</sup>) and D8 (0.1±0.1<sup>By</sup>; P<sub>TREAT</sub>=0.39, P<sub>TIME</sub><0.0001 and P<sub>TREAT\*TIME</sub>=0.0007). Also, the number of LF, unlike MAP5 group (D4: 1.3±0.4<sup>ABx</sup>, D6: 0.9±0.2<sup>Bx</sup> and D8: 2.6±0.5<sup>Ax</sup>), in FOLL group was increased over time (D4: 1.9±0.3<sup>Cx</sup>, D6: 3.9±0.8<sup>By</sup> and D8: 16.1±4.2<sup>Ay</sup>; P<sub>TREAT</sub><0.0001, P<sub>TIME</sub><0.0001 and P<sub>TREAT\*TIME</sub>=0.02). Further, an increased proportion of LF (number of MF and LF per total follicles) over time was observed for both treatments, however, the increase was more evident in the FOLL group (MAP5- D4:6.9%<sup>Bx</sup>, D6:6.0%<sup>Bx</sup> and D8:23.9%<sup>Ax</sup>; FOLL-D4:8.7%<sup>Cx</sup>, D6:19.6%<sup>By</sup> and D8:87.6%<sup>Ay</sup>; P<sub>TREAT</sub><0.0001, P<sub>TIME</sub><0.0001 and P<sub>TREAT\*TIME</sub><0.0001). Finally, ovulation rate was lower in cows from MAP5 group (33.3%) compared to FOLL group (69.1%; P=0.01). In conclusion, the association of FSH with MAP5 does not promote a superstimulation response with the same efficiency as FSH in non-lactating Holstein cows submitted to superstimulation protocols.

**Acknowledgments:** Agrindus and Tecnopec.



A096 FTAI, FTET and AI

### **Progesterone plasma concentration in non-lactating Holstein cows during reuse of intravaginal progesterone device, previously autoclaved or disinfected**

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**Keywords:** *Bos Taurus*, device, hormone.

Intravaginal devices of progestogens/progesterone (P4) have been used for decades with the aim of controlling the estrous cycle in farm animals. Although the manufacturer's recommendation be just one use, reuse has been widely reported, due to residual P4. Various ways have been used to process devices prior to reuse. However, no comparisons between different types of devices have been reported. Thus, the aim of this study was to compare plasma concentrations of P4 during the reuse of two types of intravaginal P4 devices previously autoclaved or disinfected with 8 d of use in 24 Holstein cows. Non-lactating multiparous cycling cows (~600 kg of BW and BCS 3) were used. They were kept in confinement, fed maintenance diet and water *ad libitum*. In a 2x3 factorial arrangement and two replicates, all animals underwent two treatments. Two sources of P4 (CIDR<sup>®</sup> [C], Zoetis, and Sincrogest<sup>®</sup> [S], Ourofino) and three types of processing: new (N), reused autoclaved (RA) and reused disinfected (RD) were used. The cows remained 8 d with a new device. At 7 and 8 d after device insertion, 25 mg of dinoprost (PGF<sub>2</sub>α; Lutalyse<sup>®</sup>, Zoetis) was administered and on Day 8, after the withdrawal of P4, a Norgestomet (Crestar<sup>®</sup>, MSD) ear implant was inserted, which was maintained for 24 h. On Day 9, the cows were randomized in one of six treatments (NC, RAC, RDC, NS, RAS and RDS). The devices were kept for 8 d and during this period blood samples were collected at the following times: 0, 2, 12, 24, 48, 72, 96, 120, 144, 168 and 192 h. At the last day, the P4 devices were removed and Norgestomet was inserted again, and maintained for 24 h, together with other PGF<sub>2</sub>α treatment. Then, the second replicate began. Statistical analysis was done by the Proc-mixed and the averages and standard error of P4 concentrations were calculated by the Proc-means of SAS 9.2 (P<0.05). Differences were found between the types of devices (1.34±0.04 and 1.08±0.04 ng/mL; CIDR and Sincrogest, respectively) and interaction between time and type of processing. Regarding interaction, differences were observed for RA, RD and N, respectively, at 2 h (1.58±0.23<sup>a</sup>, 0.76±0.11<sup>b</sup> and 1.07±0.11<sup>ab</sup> ng/mL), 12 h (2.09±0.20<sup>a</sup>, 1.30±0.10<sup>b</sup> and 1.75±0.17<sup>ab</sup> ng/mL), 24 h (1.97±0.16<sup>a</sup>, 1.06±0.10<sup>b</sup> and 1.42±0.10<sup>b</sup> ng/mL), 48 h (2.01±0.15<sup>a</sup>, 1.27±0.11<sup>b</sup> and 1.77±0.14<sup>a</sup> ng/mL), 72 h (1.65±0.11<sup>a</sup>, 1.16±0.11<sup>b</sup> and 1.52±0.09<sup>a</sup> ng/mL) and 96 h (1.48±0.11<sup>ab</sup>, 1.18±0.12<sup>a</sup> and 1.56±0.10<sup>b</sup> ng/mL). It is concluded that in non-lactating Holstein cows, the mean plasma concentration of P4 was higher for CIDR than Sincrogest and regardless of the type of device, the autoclaving process provided higher circulating concentrations of P4, in relation to disinfected, and similar or higher compared to the new.

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A097 FTAI, FTET and AI

### Effect of early or late resynchronization on reproductive performance of dairy cows observed for estrus

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**Keywords:** dairy cow, PAG, resynchronization.

The objective was to evaluate reproductive performance of dairy cows subjected to early (ER) or late resynchronization (LR) after nonpregnancy diagnosis. Holstein cows (n=972) were subjected to the Ovsynch protocol (D0 GnRH, D7 PGF<sub>2α</sub>, D9 GnRH, D10 AI) for first AI at 68 d in milk. Weekly cohorts of cows were blocked by parity and randomly assigned to ER, based on nonpregnancy diagnosis using pregnancy associated glycoprotein (PAG) ELISA in blood, or LR based on palpation. ER cows received GnRH 2 d before PAG testing between 27 and 33 d after the previous AI, and not reinseminated nonpregnant cows continued on the Ovsynch for timed AI (TAI). LR cows had pregnancy diagnosed by transrectal palpation between 36 and 49 d after AI and those not reinseminated nonpregnant were resynchronized with the Ovsynch starting on the day of nonpregnancy diagnosis. After the first AI, all cows were observed for estrus based on removal of tail chalk and those in heat were inseminated on the same day. The study lasted 70 d for ER and 112 d for LR to allow a maximum of two resynchronized timed AI for each treatment in cows not observed in estrus. A cow was considered pregnant at the end of the study based on palpation at 36 to 49 d after AI. Categorical and continuous data were analyzed with the GLIMMIX procedure of SAS. Time to pregnancy was analyzed using the Cox's proportional hazard model with the PHREG procedure of SAS. The sensitivity (Se), specificity (Sp), positive (PPV) and negative (NPV) predictive values of using PAG for diagnosis of pregnancy were calculated at different intervals after AI. Pregnancy per AI at first AI did not differ between treatments and averaged 28.9%. Cows in ER tended (P=0.09) to become pregnant faster after the first AI than LR cows (63 vs 73 d, respectively; adjusted hazard ratio=1.25; 95% CI=0.96-1.65). The proportion of cows resynchronized that were not pregnant to first AI was greater (P<0.01) for ER than LR (29.9 vs. 8.5%). A total of 2,129 test diagnostics for PAG were evaluated. Se (true pregnant) and Sp (true nonpregnant) of PAG for pregnancy diagnosis, according to days after AI, were: ≤ 27 d: 94.6% and 89.9%; 28-30 d: 96.1% and 90.7%; 31-35 d: 98.7% and 88.1%; > 35 d: 94.4% and 85.2%. Overall, Se was 95.1% (95% CI = 93.6-96.3), Sp was 89.0% (95% CI = 86.9-90.8), PPV was 90.1% (95% CI = 88.3-91.8), NPV was 94.5% (95% CI = 92.8-95.8), and accuracy was 92.1%. In summary, early diagnosis of nonpregnancy based on PAG with ER increased submission to TAI and tended to reduce interval to pregnancy in cows observed for estrus. The benefits of early resynchronization with a negative PAG diagnosis need to offset the 4.9% iatrogenic abortion.



A098 FTAI, FTET and AI

### Effects of reutilization of a progesterone device on follicular dynamics in ewes

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**Keywords:** hormonal protocol, ovulation, sheep.

The present study aimed to evaluate the effect of reutilization of a progesterone (P4) device for small ruminants on follicular dynamics of crossbred Santa Inês ewes in 9 days-synchronization protocol. Two consecutive replicas were performed, with nine ewes on each. The animals were randomly divided into two treatments: Control Group (CG) and Reused Group (RG). CG ewes received new intravaginal device on day 0 (D0). The device was removed on day 9 (D9) when 35µg of d-cloprostenol (Prolise<sup>®</sup>, Syntex, Argentina) and 250 IU of eCG (Folligon<sup>®</sup>, Intervet, Holland) were administrated via IM. The RG ewes were submitted to the same protocol, although the P4 device had been previously used in a similar protocol. Ultrasound exams (Chison, 9300VET, Kylumax, Brazil) were performed every 24 h from D0 to D9, and every 12 h from removal of the device until ovulation time. Statistical analyses were performed by General Linear Models (GLM) - Statistical Analyses System (SAS) after residuals normality and homoscedasticity were tested. Level of significance of 5% was considered for statistical analyses. No difference was observed between the groups on series of comparisons regarding the size of the follicle: 1) diameters of the first largest follicle (CG: 4.0±0.2 vs. RG: 4.1±0.2 mm, P=0.75) and the second largest follicle (GC: 3.1±0.1 vs. GR: 3.1±0.4 mm, P=0.83) at the moment of device removal; 2) maximum diameters of the first largest preovulatory follicle (GC: 5.7±0.2 vs. GR: 5.5±0.2 mm, P=0.09) and the second largest preovulatory follicle (GC: 4.7±0.2 vs. GR: 4.7±0.1 mm, P=0.89). Also, no difference was observed between groups on the duration of the ovulatory follicular wave duration (CG: 5.0±0.4 vs. RG: 5.0±0.5 days, P=0.93) and; in the interval between device removal and second ovulation (CG: 70.5±5.3 vs. RG: 73.2±3.0 h, P=0.67) and the day of ovulatory emergency (CG: 7.1±0.4 vs. RG: 6.8±0.6 days, P=0.76). Only significant difference between groups was observed when comparing the interval from P4 device removal to first ovulation (CG: 71.7±2.5 vs. RG: 63.9±2.7 h, P=0.05). The reutilized device had similar results on ovarian follicular dynamics of ewes, although first ovulation may occur earlier on ewes that received the reused device than on ewes with new devices.

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A099 FTAI, FTET and AI

### Relationship between bacterial isolation and somatic cell count on fertility of dairy cows receiving IVP embryos

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**Keywords:** bacterial isolation, IVF embryos, mastitis.

The aim of this study was to evaluate the relationship between bacterial isolation and somatic cell count (SCC) and its impact on maintenance of pregnancy in embryo recipient dairy cows. In experiment 1, two milk samples were collected from 2,532 Holstein and Girolando dairy cows (nine farms), with average DIM 175.1±9.2, for analysis of SCC and microbiological culture to determine bacterial growth. In experiment 2, 1,397 Girolando lactating cows with average DIM 132.9±6.2, were subjected to a fixed-time synchronization protocol for *in vitro* produced embryo (IVP) transfer (FTET). Milk samples were collected two days before the FTET for microbiological culture and SCC analysis. Pregnancy diagnosis was performed 28 days after FTET. The bacterial isolation was carried out in the Vidavet laboratory (Botucatu-SP, Brazil) and SCCS analysis was performed at the “Clínica do Leite” (Piracicaba-SP, Brazil). The animals were divided according to the bacterial isolation into 5 experimental groups according to the NMC: Gram-positive environmental (AB +; *Bacillus spp.*, *Enterococcus spp.*, *Streptococcus spp.*), Gram-negative environmental (AB-; Coliforms, *Enterobacter spp.*, *Klebsiella spp.*, *Proteus spp.*, *Pseudomonas spp.*), Gram-positive contagious (C +; *Corynebacteriumbovis*, *Staphylococcus aureus*, *Streptococcus agalactiae*), CNS (Coagulase Negative Staphylococcus) and control (no bacterial isolation). The CNS were grouped with the control group in Experiment 2 analyzes, because the isolation of these agents is not usually associated with mammary gland disease. The animals were also classified into three groups according to the SCC: less than 2x10<sup>5</sup> cells/mL, between 2x10<sup>5</sup> and 4x10<sup>5</sup> cells/mL, and greater than 4 x10<sup>5</sup> cells/mL. The SAS PROC GLIMMIX was used for statistical analysis. Results were considered significant if P<0.05 and have a tendency to differer if P<0.10. In experiment 1, the sensitivity of detection the causative agents of mastitis in samples with SCC lower than 2x10<sup>5</sup> cells/mL was 60.32%. In cows with SCC less than 2x10<sup>5</sup> cells/mL bacterial growth was detected in 27.2%, 51.4%, 38.9% and 51.4% of the samples in group C+, SCN, AB+ and AB-, respectively. In Experiment 2, there was an effect of bacteria group on the pregnancy rate (P=0.0007.) The pregnancy rate in [AB-] (30.1%; 68/201) and [AB +] (29.9%; 24/82) groups was lower than the control group (44.0; 367/869) and pregnancy rate of the [C +] (36.6%; 85/245) group tended (P=0.09) to be lower than the control group. The group with SCC greater than 400,000 cells/mL had lower pregnancy rate (P<0.01) compared to those with less than 2x10<sup>5</sup> SCC cells/mL. The group with SCC between 2x10<sup>5</sup> and 4x10<sup>5</sup> cells/mL tended (P=0.09) to have lower pregnancy rate than the group with SCC less than 2x10<sup>5</sup> cells/mL. Even in milk samples with SCC considered low (<2x10<sup>5</sup> cells/mL) isolation of the causative agents of mastitis was detected. The presence of the causative agents of mastitis decreased pregnancy rate of dairy cows.



A100 FTAI, FTET and AI

### Use of resynchronization as a tool to enhance the genetic improvement and reproductive performance in a *Bos indicus* beef cattle herd

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**Keywords:** pregnancy, resynchronization, TAI.

The economy efficiency of livestock is linked to calves production, which are destined for meat production or herd replacement. In this context, the reproductive efficiency of cows shows up as well an important objective to be achieved (Sá Filho *et al.*, 2008, 3° Simpósio Internacional de Reprodução Animal Aplicada, 54). This study aimed to investigate the reproductive performance of a *Bos indicus* beef cattle herd submitted to two TAI protocols (synchronization and resynchronization) before the first 45 days of the breeding season (BS). Thus, a total of 333 multiparous beef cows between 30 and 60 days postpartum were used. All females were subjected to the same TAI protocol at the beginning of the BS. At the beginning of the treatment (D0), the animals received an intravaginal P4 device (1.9 g P4; CIDR<sup>®</sup>, Zoetis) and 2.0 mg estradiol benzoate i.m. (Ric-BE<sup>®</sup>, União Química). On D9, the females received 7.5 mg of PGF2 $\alpha$  i.m. (Lutalyse<sup>®</sup>, Zoetis), 1.0 mg of estradiol benzoate i.m. (Ric-BE<sup>®</sup>, União Química) and 400 IU of eCG i.m. (Novormon<sup>®</sup>, MSD Animal Health), followed by removal of P4 device. The TAI was performed 48 h after the removal of the intravaginal device (D11). At the time of pregnancy diagnosis, which was carried out 30 days after TAI, the cows diagnosed as non-pregnant, were again subjected to the same TAI protocol (resynchronization). Pregnancy diagnosis of resynchronized cows was also performed 30 days after the second TAI. Pregnancy rates were calculated using the *Freq* procedure of SAS, and the difference between the rates of synchronization and resynchronization was calculated by Chi-square test ( $\chi^2$ ), with a significance level of  $P < 0.05$ . It was observed similar pregnancy rate ( $P = 0.68$ ) among cows submitted to the first TAI (51.65%, 172/333) or resynchronization (49.69%, 80/161). However, when considering the cumulative pregnancy rate of the two TAI protocols performed in sequence, it was found that before mid-breeding season (41 days) 75.68% (252/333) of females available for reproduction had conceived. Therefore, it was concluded that the technique of TAI maintains the same efficiency to both, first synchronization and resynchronization. In addition, resynchronization could serve as a tool that enhances the reproductive performance and genetic improvement, since most of the females will create products of artificial insemination.



A101 FTAI, FTET and AI

### Fixed-time transcervical artificial insemination in sheep using cooled semen

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**Keywords:** cooling, ram, spermatozoa.

Dilution allows fresh ram semen to be multiplied and used immediately in artificial insemination (AI). Cooling diluted semen to 5°C reduces the motility and metabolic activity of the spermatozoa and allows its storage for several days, which further favors its use in AI. The use of both fresh and cooled semen in AI routines presents, besides practical and economic advantages over frozen semen (Mara et al., 2005 *Theriogenology* 63, 2243-2253), greater sperm viability and better fertility rates (Wusiman et al., 2012 *Asian J. Anim. Vet. Adv.* 7, 299-308). The aim of this study was to evaluate the fertility of semen cooled up to 72 hours in Santa Inês ewes after fixed-time transcervical artificial insemination (FTTCAI). Semen samples from six rams were diluted in Glycine-yolk-milk extender (Rodello et al., 2011 *Vet. e Zootec.* 18, 239-248), packaged ( $100 \times 10^6$  spermatozoa/mL in 0.25 mL) and divided, part for immediate use as fresh semen (FS-control) and part stored at 5°C as cooled semen (CS) for 24 (CS-24), 48 (CS-48) and 72 hours (CS-72). A total of 242 nulliparous and multiparous ewes underwent estrus synchronization protocol (Biscarde et al., 2010 *Rep. Fert. Develop.*, 22, 376-377): first day of synchronization (D0), 45 µg of prostaglandin; D3, insertion of sponge impregnated with 60 mg of medroxyprogesterone acetate; D7, sponge removal, 400 IU of equine chorionic gonadotropin and detection of estrus by teasing every 12 hours until the moment of AI; D8, 25 µg of gonadotropin-releasing hormone. The AI was performed, on average, 52 hours after sponge removal with use of cervical traction and Aplicador Expansor Ovino<sup>®</sup> (Alta Genetics, Brazil). Pregnancy rate and prolificacy data were analyzed by chi-square and Kruskal-Wallis test, respectively, both at a significance level of 5%. The overall pregnancy rate obtained (29%) was not significantly affected ( $P>0.05$ ) by the semen cooling time: FS = 30%; CS-24 = 23%; CS-48 = 21%; and CS-72 = 26%. The semen cooling time did not affect ( $P>0.05$ ) prolificacy (160%). It was concluded that it is possible to maintain ram semen fertility for at least three days under cooling at 5°C, enabling this practical technology to be used in semen transportation for artificial inseminations over long distances.



A102 FTAI, FTET and AI

### Different hormonal stimulus during synchronized proestrus alter the ovarian follicle responses and subsequent luteal function in suckled anestrus zebu cows

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**Keywords:** estradiol, progesterone, reproduction.

The present study evaluated the effects of treatment with estradiol cypionate (ECP) or equine chorionic gonadotropin (eCG) during the synchronized proestrus on follicular and luteal characteristics and pregnancy rates in suckled anestrus zebu cows. A total of 172 suckled Nelore cows, received a used intravaginal progesterone (P4) device (Sincrogest<sup>®</sup>, Ouro Fino Agribusiness) and 2 mg im estradiol benzoate (Sincrodiol<sup>®</sup>, Ouro Fino Agribusiness). Eight days later, devices were removed and cows received 0.25 mg i.m. of cloprostenol (Sincrocio<sup>®</sup>, Ouro Fino Agribusiness). At this moment, females were randomly assigned into one of four treatments (control, ECP, eCG and ECP+eCG) in a 2x2 factorial design. Cows in the control group received no further treatment, while cows in the ECP group received 1mg ECP (ECP<sup>®</sup>, Zoetis Brazil) and those in the eCG group received 400 IU eCG (Folligon<sup>®</sup>, MSD Animal Health) at the same moment of the P4 device removal. Cows in the ECP+eCG group received both treatments at the same time previously mentioned. Cows had their tailhead painted using chalk marker at the time of P4 device removal to evaluate the occurrence of estrus. Immediately before TAI, all the cows were treated with 10µg im busserelin (Sincroforte<sup>®</sup>, Ouro Fino Agribusiness). Plasma P4 concentration (ng/mL) was evaluated 7 days after GnRH treatment. Data were analyzed using SAS Proc GLIMMIX. There was no interaction between treatments in any response variables ( $P > 0.05$ ). The DF diameter at device removal was similar between eCG (11.2±0.3 mm, n=86) and no eCG groups (10.9±0.3, n=86  $P=0.38$ ). However, an increased DF diameter at TAI (12.6±0.3 vs. 13.5±0.3 mm,  $P=0.03$ ), dominant follicle growth rate (mm/day) from P4 device removal to TAI (0.9±0.1 vs. 1.2±0.1 mm,  $P=0.01$ ), occurrence of estrus (46.4% vs. 63.7%;  $P=0.03$ ), ovulation rate (82.6% vs. 96.7%,  $P=0.008$ ), diameter of corpus luteum (CL; 18.2±0.4 vs. 19.4±0.4 mm,  $P=0.04$ ) and plasma P4 concentration (3.9±0.2 vs. 4.8±0.2;  $P=0.001$ ) was observed in the eCG group. Though, the eCG treatment did not affect the pregnancy per AI (P/AI; 36.9% vs. 43.1%,  $P=0.42$ ). Similarly, the DF diameter at P4 device removal was similar between ECP (11.1±0.3, n=85) and no ECP groups (11.1±0.3, n=87;  $P=0.90$ ). Furthermore, the ECP treatment did not affect the DF diameter at TAI (13.0±0.3 vs. 13.1±0.3,  $P=0.90$ ), dominant follicle growth rate (mm/day) from P4 device removal to TAI (1.1±0.1 vs. 1.0 ± 0.1,  $P=0.52$ ), ovulation rate (90 6% vs. 93.6%,  $P=0.54$ ), CL diameter (19.0±0.4 vs. 18.6±0.4,  $P=0.54$ ) and plasma P4 concentration (4,4±0,2 vs. 4,4±0,2;  $P=0,94$ ), however increased occurrence of estrus (44.6% vs. 65.4%,  $P=0.008$ ) and P/AI (33.2% vs. 47.1%,  $P=0.07$ ) was observed (no eCG and eCG groups, respectively). It is concluded that treatment with eCG but not with ECP given at the time of removal of P4 device can alter ovarian follicular response and subsequent luteal function in suckled anestrus zebu cows. Moreover, both treatments (eCG or ECP) increase the number of suckled cows displaying estrus following the synchronization protocol.

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A103 FTAI, FTET and AI

### **Leptospirosis serological identification and the possible interference of the disease in FTET protocols efficiency and in reproductive parameters of embryo recipient cows**

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**Keywords:** embryos, leptospira, serologic test.

Leptospirosis is an acute infectious disease, zoonotic, cosmopolitan, which affects several species of domestic animals, and wild rodents. The incidence of leptospirosis is highest in tropical regions particularly during the rainy seasons. The possibility of its occurrence also in the state of Acre is not ruled out, though, to date, has not been diagnosed or reported before. A total of 235 crossbred cows (*Bos taurus* x *Bos taurus indicus*) as embryo recipients, subjected to identical synchronization of ovulation protocol for fixed time embryo transfer (FTET) were used. On the 16th day after the beginning of the protocol, an *in vitro* produced embryo was transferred to each recipient and, at the same time it was collected from each recipient a sample of blood by venous puncture of the coccygeal vein in tubes with vacuum without anticoagulant that were subsequently processed in attempt to obtain serum. The microscopic agglutination test (MAT) was used as diagnostic way in the presence of at least 18 leptospiras serovars grown in the laboratory. Pregnancy diagnosis and reevaluation of recipients pregnant for confirmation of pregnancy or abortion were performed on the 25th and 55th days after FTET, respectively both by ultrasonography (Aloka SSD 550, Aloka, Japan). Serological diagnosis was performed by R & D Center of Animal Biological Institute of São Paulo and statistical analysis used was the chi-square test at a significance level of 5%. As a result of testing, animals were identified 128 (128/235) reagents for at least one of serovars and 107 (107/235) of total non-reactive worked. Of the 158 recipients who responded to hormonal protocol (in D16, the presence of CL), 72 were positive and 86 were not, while of the 77 non-responders, 51 were positive and 26 were not. Of the 54 pregnant recipients, 27 were positive and of the 11 abortions observed on the 55th day after FTET, 3 were among reagents recipients for the following serovars (value of the titer): *L. icterohaemorrhagiae* (200), *L. wolffi* (200) and *L. hardjo* (400), one animal; *L. wolffi* (400) and *L. hardjo* (400) one animal; *L. wolffi* (1600) and *L. hardjo* (1600), one animal. It is concluded that the rate of utilization of protocols to FTET was dependent, statistically, on the recipient serum reagent condition ( $P < 0.05$ ), interfering in ovulation process. However, further studies are necessary to explain possible mechanisms of action of leptospirosis in the transfer/treated rate of FTET protocols.



A104 FTAI, FTET and AI

### **Conception rate in Nelore cows synchronized with different protocols of ovulation induction**

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**Keywords:** females, FTAI, inducers.

Strategies have been developed to improve the responsiveness and efficiency of hormonal protocols, seeking a synchronized ovulation and better reproductive rate. The association of ovulation induction can be used to better timing of ovulation and improves in response to hormone treatments. Therefore, this study aimed to evaluate the effect of different protocols of ovulation induction about conception rate of Nelore cows submitted to a program of FTAI. Therefore, 196 cows multiparous Nelore were submitted to following protocol: At a random day of the estrous cycle, called day 0 (D0), they received an intravaginal progesterone device (PRIMER<sup>®</sup>, Tecnopec, São Paulo, Brazil) and an application of 2mg of estradiol benzoate (RIC-BE<sup>®</sup>, Tecnopec) intramuscularly (im). On D8, the intravaginal progesterone device was removed and 150µg of d-cloprostenol (Prolise<sup>®</sup>, Tecnopec) im. plus 10mg of FSHp (Folltropin<sup>®</sup>, Tecnopec) im were administered. At this time, the animals were assigned into three groups according to the protocol for synchronization of ovulation: Group EB (n = 62) - the animals received 1mg of estradiol benzoate (RIC-BE<sup>®</sup>, Tecnopec) im. on D8; Group GnRH (n = 62) - the animals received 25mg Lecirelin acetate (Gestran<sup>®</sup>, Tecnopec) on D10; and EB + GnRH group (n = 72) - the animals were treated with 1mg of estradiol benzoate on D8 and 25µg of Lecirelin acetate on D10. On D10 of the protocol, all animals were inseminated. The pregnancy diagnosis was performed by transrectal ultrasound 30 days after FTAI using 6.0MHz linear transducer (Pie-Medical, Falcon 100, Maastricht, The Netherlands). Statistical analysis was performed using SPSS, version 19. The conception rate was compared among groups using Chi-square ( $\chi^2$ ), considering 5% as significance level. The overall conception rate was 62.0%. There was no significant difference among experimental groups on the conception rates [EB (59.71%), GnRH (61.32%) and BE + GnRH (54.23%)]. We conclude that the application of oestradiol benzoate, GnRH or association between these drugs promotes similars conception rates in females submitted to a FTAI protocol, demonstrating that all three synchronization of ovulation protocols can be used effectively in FTAI programs in Nelore cattle.



A105 FTAI, FTET and AI

### Strategies to increase fertility in lactating dairy cows submitted to TAI and FTET E2/P4 based protocols

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**Keywords:** fixed-time artificial insemination, fixed-time embryo transfer, progesterone.

The objective was to evaluate strategies to improve fertility in dairy cows submitted to timed artificial insemination (TAI) and fixed-time embryo transfer (FTET) on estradiol (E2)/progesterone (P4)-based protocols. The experiment was performed in farms that use TAI (4, n=1,642) and FTET (6, n=1,055). The animals were randomly assigned to receive one of the following treatments: (control) D-11 2mg BE (Estrogen<sup>®</sup>, Farmavet, SP-Brazil) + CIDR (CIDR<sup>®</sup>, Zoetis, SP, Brazil); D-4 PGF (Lutalyse<sup>®</sup>, Zoetis, SP-Brazil); D-2 CIDR + 1 mg ECP (ECP<sup>®</sup>, Zoetis, SP, Brazil); D0 AI or D7 ET; (2PGF) the same protocol control with two doses of PGF, the first on D-4 and second on D-2, (GnRH) the same protocol 2PGF, with GnRH (Cystorelin<sup>®</sup>, Merial, SP, Brazil) in D-11. The PROC GLIMMIX was used to evaluate the binomial variables and PROC MIXED to evaluate continuous variables. Was considered significant when  $P < 0.05$  and trend when  $P < 0.1$ . The protocol with GnRH increased the proportion of cows with a corpus luteum (CL) and progesterone (P4) in D-4 (69%,  $2.88 \pm 0.28 \text{ ng/mL}$ ) compared to control (52%,  $2.50 \pm 0.28 \text{ ng/mL}$ ) and 2PGF (51%,  $2.53 \pm 0.28 \text{ ng/mL}$ ). There was an effect of protocol 2PGF (88%) and trend of GnRH (87%) to increase the proportion of cows with CL in D7, compared to control (84%). In cows with CL on D7, the 2PGF protocol resulted in greater P4 on D7 ( $2.68 \pm 0.2 \text{ ng/mL}$ ) compared to GnRH ( $2.50 \pm 0.2 \text{ ng/mL}$ ) and control ( $2.55 \pm 0.2 \text{ ng/mL}$ ). At 32d to the protocol GnRH resulted in higher P/IA (40%) compared to control (32%) and tended to have higher P/AI compared to 2PGF (35%). At 60d the protocol with GnRH showed higher P/IA (33%) compared to control (26%), the 2PGF group not differs from the others (29%). In cows with CL in D7 the protocol GnRH had higher P/AI at 32 and 60d (44 and 36%) compared to control (35.5 and 30%), the 2PGF group not differs from the others (40 and 33.5%). In FTET, there was no difference between treatments at 32 and 60d (control=44 and 35; 2PGF=43 and 35; GnRH =43 and 39%). In cows with CL on D-4 that had  $P4 > 1.0 \text{ ng/mL}$  at D7 the P4 at D0 with the highest accuracy for pregnancy at 60d was  $\leq 0.13 \text{ ng/mL}$ . Cows with  $P4 \leq 0.13 \text{ ng/mL}$  at D0 showed the best P/IA (44%) and P/TE (41%) in relation to  $P4 > 0.13 \text{ ng/mL}$  (IA = 36, TE = 25%). In cows with CL at D-4, the protocols GnRH and 2PGF resulted in lower P4 at D0 (GnRH= $0.17 \pm 0.05$ ; 2PGF= $0.19 \pm 0.05 \text{ ng/mL}$ ), compared to control ( $0.35 \pm 0.05 \text{ ng/mL}$ ). Higher P4 at D-4 resulted in greater P/AI at 60d, but there was no effect of P4 at D-4 on P/TE. Cows with CL at D7 that displayed estrus had a higher P/IA (35%) and P/TE (39%) at 60d, compared to cows that did not display estrus (IA=25, TE=25%). The use of GnRH D-11 combined to the use of two doses of PGF increased P/AI in cows receiving E2/P4-based protocols, and the mechanism to this result is probably associated with increased P4 concentration during follicle development



A106 FTAI, FTET and AI

### The seasonal anestrus does not reduce ovarian responses in dairy buffaloes submitted to synchronization of ovulation protocols for TAI during nonbreeding season

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**Keywords:** artificial insemination, breeding season, follicles.

The aim of this study was to compare ovarian follicular and corpus luteum dynamics in dairy buffaloes submitted to timed artificial insemination (TAI) during the breeding and nonbreeding season. Thirty one lactating dairy buffaloes cows raised exclusively on grazing from farm in the Ribeira Valley, State of São Paulo, were used. The females were synchronized by the same TAI protocol during the breeding season (n=16; autumn-winter) or out of the breeding season (n=15, nonbreeding season, spring-summer). On D0, cows received a new intravaginal progesterone (P4) device (1 g of P4; Sincrogest<sup>®</sup>, Ourofino Agronegócio) associated with 2.0 mg of estradiol benzoate (Sincrodiol<sup>®</sup>, Ourofino Agronegócio). At this time, females were classified according their body condition score (BCS; scale 1-5). On D9 (PM) cows received i.m. 0.53mg of PGF2 $\alpha$  (Cloprostenol, Sincrocio<sup>®</sup>, Ourofino Agronegócio) and 400 IU of eCG (Novormon<sup>®</sup>, MSD Animal Health), followed by P4 device withdraw. On D11 (PM) 10  $\mu$ g of buserelein acetate (GnRH, Sincroforte<sup>®</sup>, Ourofino Agronegócio) was i.m. administrated. The TAI was performed 16 hours after GnRH treatment (D12; AM). The ultrasound evaluations (Mindray DP2200Vet, China) were performed on D0 and D9 to verify the cyclic status (CL presence). Also ultrasound exams were done on D9, D11 and D12 to verify the size (diameter) and growth of dominant follicle (DF). The corpus luteum (CL) diameter was evaluated on 6, 10 and 14 days after TAI. Pregnancy diagnosis was performed 30 and 45 days after TAI. Continuous variables were presented as mean  $\pm$  standard error (SEM). The variables were analyzed by *Glimmix* procedure of SAS<sup>®</sup> and P values <0.10 were considered different. Cows synchronized during the breeding season presented more days in milk (DIM; 103.0 $\pm$ 10.9 vs. 51.9 $\pm$ 4.68 days; P=0.0002), and higher percentage of CL (81.25 vs. 0.0%; P=0.02) at onset of synchronization protocol. However lower BCS was observed in cows synchronized during the Breeding season than those synchronized during the nonbreeding season (3.1 $\pm$ 0.1 vs. 3.7 $\pm$ 0.1; P=0.02). There was no differences between the seasons on DF at D11 (11.0 $\pm$ 0.8 vs. 13.2 $\pm$ 0.5 mm; P=0.12), growth of DF (1.6 $\pm$ 0.2 vs. 2.6 $\pm$ 0.3 mm/day; P=0.35), CL at D18 (19.8 $\pm$ 1.2 vs. 17.4 $\pm$ 0.5 mm; P=0.20), CL at D26 (23.1 $\pm$ 1.3 vs. 19.3 $\pm$ 0.7 mm; P=0.12), on pregnancy at D42 (43.8 vs. 66.7%; P=0.1589) and D57 (43.8 vs. 66.7%; P=0.16). However higher DF on D9 (7.9 $\pm$ 0.7 vs. 8.7 $\pm$ 0.6 mm; P=0.09), FD on D12 (11.3 $\pm$ 0.8 vs. 14.0 $\pm$ 0.5 mm; P=0.08), and ovulation rate (68.7 vs. 93.3%; P=0.06) was found at nonbreeding season than at breeding season. The CL on D22 (24.1 $\pm$ 1.0 vs. 18.0 $\pm$ 0.5 mm; P=0.03) were largest at breeding season. Therefore, it was concluded that seasonal anestrus does not reduce the ovarian responses in lactating dairy buffalo cows submitted to TAI protocols during nonbreeding season, corresponding to the period that females had greater BCS.

**Acknowledgments:** Ourofino Agronegócio.



A107 FTAI, FTET and AI

### **Effect of inhibition of angiotensin converting enzyme (ACE) on relaxing degree of the cervix in goats submitted to fixed-time artificial insemination**

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**Keywords:** enalapril, goat, uterus.

In order to improve the reproductive rates in species of economic interest, the enalapril, an ACE inhibitor, has been used as a tool in the growth of reproductive biotechnologies, after discovering a new peptide involved with reproduction: the presence of a new component of the renin-angiotensin system in ovaries of rats, Angiotensin-(1-7) (Costa et al. 2003), whose production is increased by inhibition of ACE. The aim of this study was to evaluate the effect of ACE inhibition on relaxing of the cervix in goats subjected to fixed-time artificial insemination (FTAI). Ninety four goats were undergone to estrus synchronization (D0) with intravaginal sponges (60 mg, MAP) for 12 days (D0-D12) and intramuscular injection of 300 IU of eCG and 75µg of PGF2α on the tenth day of treatment (D10). In D10, the goats were divided into three groups: G1 (n = 34) Control, G2 (n = 30) enalapril maleate in the formulation of vaginal tablets, and G3 (n = 30) were given subcutaneously 3 mL of enalapril maleate suspended in oil (20 mg/mL). There were two inseminations with fresh semen (50x10<sup>6</sup> spermatozoa/insemination dose), collected from reproducers of proven fertility, diluted in coconut water, the first being held 36 hours after sponge removal and the second 12 hours after the first insemination. The relaxation of the cervix was measured by the easiness of pipette passage by cervical rings, being classified as contracted (C) when the pipette passed the first three rings, semi-relaxed (SR) when the pipette surpassed until the sixth ring and relaxed (R) when the pipette achieved the uterine lumen. The results were tested with  $\chi^2$  (chi-square  $p \leq 0.05$ ) using SAS 9.0. In the first insemination, the best cervix relaxation represented by the largest percentage of intrauterine inseminations, was observed in G2: G1 [C=70.59% (24/34), SR=23.53% (8/34) and R=5.88% (2/34)]; G2 [C=50.00% (15/30), SR=26.67% (8/30) and R=23.33% (7/30)]; and G3 [C=70.00% (21/30), SR=23.33% (7/30) and R=6.67% (2/30)]. In the second insemination the degree of relaxation was worse in G3 represented by the lower percentage of intrauterine insemination: G1 [C=32.35% (11/34), SR=17.65% (6/34) and R=50.00% (17/34)]; G2 [C=26.67% (8/30), SR=26.67% (8/30) and R=46.66% (14/30)]; and G3 [C=46.67% (14/30), SR=20.00% (6/30) and R=33.33% (10/30)]. In conclusion, this study suggests that enalapril administered via intravaginal can anticipate the relaxation of the cervix of goats subjected to FTAI.



A108 FTAI, FTET and AI

### Equine chorionic gonadotropin effect on the occurrence of estrus and pregnancy rates of primiparous and multiparous *Bos indicus* cows submitted to TAI

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**Keywords:** eCG, fertility, zebu.

The objective of this study was to evaluate the effect of equine chorionic gonadotropin (eCG) treatment on the occurrence of estrus and pregnancy rate in *Bos indicus* primiparous and multiparous cows submitted to timed artificial insemination (TAI). The study enrolled 603 lactating cows (288 primiparous and 315 multiparous; with 30 to 60 days post-partum) with body condition score (BCS) of  $2.75 \pm 0.01$  (1 to 5 scale). On a random day of the estrus cycle (D0) all animals received 2 mg of estradiol benzoate (Sincrodiol®, Ourofino, Brazil) and an intravaginal progesterone device (Sincrogest®, Ourofino, Brazil). On D8 all cows received 150µg of cloprostenol (Sincrocio®, Ourofino, Brazil), 1 mg of estradiol cypionate (ECP®, Pfizer, Brazil) and the progesterone devices were removed. Still on D8, the females were randomly assigned to one of two treatments (Control Group and eCG Group). Animals from eCG group received 300UI of eCG (Folligon, MSD, Brazil) and cows from Control group received no additional treatment. The cows were inseminated at fixed time, 48 hours after progesterone device removal. Pregnancy diagnosis was performed 35 to 45 days after the TAI. Estrus detection was performed using a chalkmarker on the tailhead. All data were analyzed by GLIMMIX procedure of SAS. Regarding the occurrence of estrus, there was no interaction treatment and category ( $P=0.27$ ), treatment effect [control group 60.3% (190/315) and eCG group 66.0% (190/288);  $P=0.17$ ] and animal category effect [Primiparous 60.8% (175/288) and multiparous 65.1% (205/315),  $P=0.26$ ]. However, cows that displayed estrus following the TAI protocol had higher pregnancy rate [Cows that displayed estrus, 41.1% (153/372) and that did not display estrus 24.6% (50/219)]. For pregnancy rate, there was an interaction treatment and animal category ( $P=0.005$ ). Treatment with eCG increased ( $P=0.0001$ ) pregnancy rate in both primiparous cows [control group 9.7% (15/154) and Group eCG 41.4% (55/133)] and multiparous cows [Control group 37.5% (60/160) and group eCG 49.7% (77/155)]. However, the difference between treatments was more evident in primiparous cows. Moreover, primiparous cows [24.4% (70/287)] had lower pregnancy rate than multiparous cows [43.5% (137/315);  $P=0.001$ ]. Thus, despite the administration of eCG in TAI protocols do not interfere in the estrus occurrence, the eCG is essential to increase the pregnancy rate, especially in primiparous cows.



A109 FTAI, FTET and AI

### **Resynchronization 22 days after the first TAI does not alter the pregnancy loss in non-lactating zebu cows**

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**Keywords:** bovine, estradiol, reproduction.

Preliminary studies have shown that replacement of GnRH by estradiol benzoate (EB) at the beginning of the resynchronization protocol 22 days after the first timed artificial insemination (TAI), with no previous pregnancy diagnosis, determines acceptable conception rates. However, it is still not established whether the administration of 1 mg of EB, 22 days after the first TAI, could increase the pregnancy loss of pregnant females at this treatment. Thus, the present study aimed to evaluate the occurrence of pregnancy loss in non-lactating beef cows submitted to resynchronization 22 days after the first TAI (RE) or submitted only to natural mating (NM) 15 days after the first TAI. Therefore, 351 cows (112 Nellore and 239 crossbred cows) were randomly assigned in two experimental groups: RE (n = 178) and NM (n = 173). All animals were subjected to a same synchronization of ovulation protocol for the first TAI, which consisted of inserting an intra-vaginal progesterone device (P4; DIB, MSD Animal Health) previously used for eight days plus 2 mg of estradiol benzoate (EB; Gonadiol, MSD Animal Health). After eight days, the intravaginal devices were removed and the females received Sodic Cloprostenol (0.265 mg; Ciosin, MSD Animal Health), 1 mg of Estradiol Cypionate (ECP, Zoatis Animal Health) plus 300 IU of eCG (Folligon, MSD Animal Health). The cows received a TAI 48 hours after intravaginal devices removal. The females of the NM group were maintained at a 1:25 bull-cow proportion, from 15 to 90 days after the first TAI. The females from RE group were synchronized again 22 days after the first TAI. The same protocol previously described was used, except by the use of a norgestomet auricular implant (3 mg; Crestar; MSD Animal Health) and the EB dosage at the implant insert (1 mg; Gonadiol, MSD Animal Health). In the latter group the bulls were introduced 10 days after the second TAI. The pregnancy diagnosis was performed by ultrasonography 30 days after each TAI and after the end of the breeding season (120 days after the first TAI). No differences were found on pregnancy rates at first TAI protocol (NM=63.0%; 109/173 vs. RE=68.5%; 122/178; P=0.12) and on pregnancy rates after the end of breeding season (NM=95.4%; 165/173 vs. RE=93.8%; 167/178; P=0.41). The pregnancy rate of the resynchronization was 46.4% (26/56). Also, there was no difference (P=0.40) on the pregnancy loss between 30 and 60 days of resynchronized females (RE=2.5%; 3/122) or only NM (NM=0.9%; 1/109). It was concluded that the use of resynchronization at 22 days after the first TAI, without previously diagnosis, does not alter the pregnancy loss of non-lactating zebu females.

**Acknowledgments:** MSD Animal Health; Agropecuária Estrela do Céu Ltda.



A110 FTAI, FTET and AI

## The use of sorted sperm in dairy cows under different reproductive managements

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**Keywords:** dairy cows, reproduction, reproductive efficiency.

The aim of this study was to evaluate the reproductive efficiency of dairy cows subjected to synchronization of ovulation protocol for timed artificial insemination (TAI) or artificial insemination after estrus detection (ED+AI) using sex-sorted sperm. A total of 626 crossbreed (Gir x Holstein) and Holstein lactating cows, receiving the 1<sup>st</sup> to 3<sup>rd</sup> service, producing 23.8±0.4 Liters of milk/day, and with 77.0±1.7day in milk were used. At onset of the program, cows that had corpus luteum (CL) by ultrasonography exam (U.S.) were randomly assigned to one of two groups (ED+AI or TAI/CL). Females that presented absence of CL at that moment were synchronized and were timed inseminated (TAI/noCL). The females from ED+AI were inseminated 12 h after estrus detection during six consecutive days after 500 mg im of cloprostenol (PGF2, Ciosin<sup>®</sup>, MSD Animal Health) administration plus an adhesive aid to detection of estrus (Estrotec<sup>®</sup>, Brazil IVP). Cows from TAI groups (CL or noCL) received a synchronization of ovulation protocol. At onset of the treatment cows received an intravaginal progesterone device (P4; DIB<sup>®</sup>, new or previously used for 8 days, MSD Animal Health) plus 2 mg i.m. of estradiol benzoate (EB; Gonadiol<sup>®</sup>, MSD Animal Health). At P4 device removal (8 days after insertion), cows received a dose of PGF2 $\alpha$  and 1 mg i.m. EB was administered 24 hours later. The TAI was performed 60 hours after P4 device removal. Pregnancy diagnosis was performed 32±3 days after insemination. Statistical analysis was performed by orthogonal contrast using PROC GLIMMIX of SAS. The analyzed contrasts were: 1) Type of reproductive management: ED+AI vs. TAI (CL or noCL), and 2) The presence of CL at onset of synchronization: TAI CL vs. TAI noCL. Cows receiving ED+AI had lower service rate (ED+AI= 45.1%, 101/224; TAI CL= 94.2%, 180/191; and TAI noCL= 97.2%, 205/211; P<0.0001); however, higher conception rate (ED+AI= 31.7%, 32/101; TAI CL= 19.4%, 35/180; and TAI noCL= 23.9%, 49/205; P=0.03] than cows receiving TAI. Still, there was a tendency to enhance the pregnancy rates in cows receiving TAI than cows inseminated after estrus detection (ED+AI=14.3%, 32/224; TAI CL= 18.3%, 35/191; and TAI noCL= 23.2%, 49/211; P=0.07). There was no effect of the presence of CL at onset of the synchronization protocol on conception (P=0.45) or pregnancy (P=0.28) rates. Thus, it is concluded that TAI programs increase the service rate of lactating dairy cows, regardless of the presence of CL at beginning of the treatment. Furthermore, conception rate achieved after TAI using sexed semen, regardless of the presence of CL in the beginning of the synchronization protocol, is lower than that obtained upon detection of estrus in lactating dairy cows.



A111 FTAI, FTET and AI

## TAI anticipation in buffaloes submitted to ovulation synchronization during the non breeding season

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**Keywords:** anestrus, estradiol benzoate, induction.

The timed artificial insemination (TAI) anticipation in buffaloes submitted to ovulation induction with estradiol benzoate (EB) during the non breeding season was evaluated. The hypothesis was that early TAI increases the pregnancy rate of females submitted to ovulation induction with EB. In this study, 204 buffaloes were distributed according to age, number of births, the body condition score and ovarian activity in one of two groups (Group TAI 64h, n=101 and Group TAI 56h, n=103). At a random day of the estrous cycle (D0), in the morning (Group TAI 56h) or afternoon (Group TAI 64h), buffaloes received an intravaginal progesterone device (P4; Sincrogest<sup>®</sup>, Ourofino Agronegócio, Brazil) and 2mg im of EB (SincrodioI<sup>®</sup>, Ourofino Agronegócio). On D9 AM (Group TAI 56h) or PM (Group TAI 64h), females received 530µg im of Cloprostenol sodium (Sincrocio<sup>®</sup>, Ourofino Agronegócio) and 400IU of eCG (SincroeCG<sup>®</sup>, Ourofino Agronegócio), followed by P4 device removal. On D10 AM (Group TAI 56h) or PM (Group TAI 64h), buffaloes received 1mg im of EB (SincrodioI<sup>®</sup>, Ourofino Agronegócio). The buffaloes of TAI 56h group and TAI 64h group were timed inseminated 56h and 64h after the P4 device removal (D11, PM and D12, AM, respectively). Females were submitted to ultrasonographic evaluation (Mindray DP2200Vet, China) on D0 to assess ovarian activity (presence of corpus luteum) and on D42 for the pregnancy diagnosis. A subset of animals (TAI 64h group, n=48 and TAI 56h group, n=47) was submitted to ultrasonography exam to evaluate the dominant follicle diameter (DF) at the time of TAI. The statistical analysis was performed using the GLIMMIX procedure of SAS<sup>®</sup>. There was no difference between experimental groups (TAI 64h and TAI 56h) on DF at the time of TAI ( $12.5 \pm 0.4$  vs.  $12.9 \pm 0.3$  mm) and on the pregnancy rate [52.5% (53/101) and 49.5% (51/103)], respectively ( $P > 0.05$ ). However, it was found effect of the DF on pregnancy rate ( $P < 0.02$ ), being 8.3% (1/12)<sup>b</sup> to DF <10.0 mm; 26.1% (6/23)<sup>b</sup> to DF between 10.1 to 12.0 mm; 68.9% (31/45)<sup>a</sup> and DF > 12.1 mm. The hypothesis of this study was rejected. It is concluded that the anticipation of TAI in buffaloes submitted to ovulation induction with EB during the non breeding season do not change the fertility of these females. However, it is possible to perform TAI during entire time of the day (AM and PM) in buffaloes synchronized during the non breeding season.



A112 FTAI, FTET and AI

### Effect of supplementation of progesterone after AI in corpus luteum formation and conception rate of dairy cows

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**Keywords:** *Bos taurus*, fertility, hormone.

In order to evaluate the effect of supplementation of progesterone (P4) in the formation of the corpus luteum (CL; Exp 1), the pregnancy to AI (Exp 2) and ET (Exp 3) experiments were carried out. In Exp 1, 42 Holstein cows were divided into two groups: CtrlAI (n=21) and SP4AI (n=21; intravaginal P4 device with 1.9g, CIDR, Zoetis) 3 d after AI. The fixed-time artificial insemination (FTAI) protocol used consisted of placing a CIDR and 2mg estradiol benzoate (EB; Gonadiol, MSD, im, D -10). On D -3, 25mg dinoprost (Lutalyse, Zoetis, im) and D -2, 1mg estradiol cypionate (ECP, Zoetis, im) with CIDR removal. On D 3, CIDR was inserted in group SP4AI and removed after 17 d (D 20). Blood samples were collected on D 3 (before CIDR insertion in group SP4AI), 4, 7, 11, 14, 17, 30 and 31 for plasma concentration of P4 by RIA. Ultrasound scans were performed at 4, 7, 11, 14 and 20 after FTAI to calculate the CL volume. In Exp 2, 668 Holstein cows and crossbreds were divided into the same groups of Exp 1 and subjected to the following FTAI protocol: CIDR insertion and 2mg of EB or 0.1mg Gonadorelin (Fertagyl, MSD, im), D -10. On D -3, 25mg of dinoprost (Lutalyse). On D -1.5, CIDR removal, 1mg EB and Lutalyse. AI occurred on D 0. CIDR after AI remained in the cows for 14 d (D 3 to 17). Pregnancy diagnoses occurred at 31 and 90 d after AI. In Exp 3, 360 Holstein cows were divided into three groups: CtrlET (n=132); SP4ET4 (n=119), CIDR was placed 4 d before ET and removed on the day of ET, and SP4ET14 (n=109), CIDR was placed 4 d before ET and removed 14 d after insertion. The recipients received Lutalyse 10 d before ET and estrus was observed. In vitro-produced embryos were transferred to cows on Day 7, 8 or 9 after estrus. Statistical analysis was performed using the GLIMMIX procedure of SAS (P<0.05). In Exp 1 there was Trt x Day interaction related to greater plasma concentration of P4 due to supplementation (Day 4: 0.9±0.2 vs. 2.2±0.2; and Day 7: 2.7±0.2 vs 3.6±0.2 ng/mL). In relation to CL, there was no Trt x Day interaction or treatment effect, with only effect of day. In Exp 2, there was no effect of supplementation of P4 on conception rates at 31 (32.2 vs. 31.5% [CtrlAI and SP4AI]) or 90 (26.6 vs. 24.6%) days after AI, or embryonic loss (16.4 vs. 18.0%). In Exp 3, there was no effect of day of the estrous cycle of the recipient or Trt x Day interaction. However, the treatment decreased the pregnancy per ET, both in cows supplemented for 4 or 14 d at Day 31 (39.6 vs. 21.0 vs. 14.7% [CtrlET, SP4ET4 and SP4ET14]) or Day 90 (26.7 vs. 11.0 vs. 10.0%), with no effect on embryonic loss (31.0 vs. 43.9 vs. 22.2%). Therefore, supplementation with a P4 device on Day 3 after FTAI does not seem to impair the development or luteal function, but also does not increase conception in lactating cows submitted to FTAI. However, supplementation with P4 decreased pregnancy per ET in embryo recipients.

**Acknowledgments:** CAPES, CNPq, FAPESP, Zoetis and Rehagro.



A113 FTAI, FTET and AI

### The reduction of proestrus period changes the occurrence of estrus, but does not affect follicular development and luteal function in Nelore cows synchronized for TAI

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**Keywords:** bovine, estradiol, progesterone.

The aim of the present study was to evaluate the effects of exogenous estradiol supplementation and the reduction of proestrus period (48 vs. 24 hours) in zebu cows. Lactating cycling (i.e. with a CL) pluriparous cows (n=47) with a body condition score (BCS) 3.68±0.04 were used. Cows were synchronized using an intravaginal progesterone (P4) device containing 0.6 g of P4 (Cronipress Monodose M-24<sup>®</sup>, Biogênese-Bagó, Paraná, Brasil) plus 2 mg of estradiol benzoate (Sincrodiol<sup>®</sup>, Ouro Fino Agronegócio). Eight days later, cows received 0.25 mg of cloprostenol (PGF2 $\alpha$ , Sincrocio<sup>®</sup>, Ouro Fino Agronegócio) and were randomly assigned into one of three groups. The Control (n=15) and ECP (n=17) groups had the P4 devices removed at the time of PGF2 $\alpha$  administration, whereas the short proestrus period treated cows (SHORT; n=15) had the P4 devices removed 24 hours later. In addition, ECP treated cows received 1mg de ECP (ECP<sup>®</sup>, Zoetis Brasil) at the P4 removal. Cows from all groups also received an Estrotec<sup>®</sup> to evaluate the estrus occurrence and 10 $\mu$ g i.m. of buserelin acetate (GnRH, Sincroforte<sup>®</sup>, Ouro Fino Agronegócio) was administrated 48 hours later. Thus, control and ECP treated cows had a 48 hours proestrus and the SHORT treated cows had 24 hours of proestrus. Plasma progesterone concentration was measured seven days after GnRH injection (ng/mL). Ultrasound exams were performed: 1) at the onset of the protocol (cyclicity); 2) on the eighth day of treatment, i.e., at time of P4 device removal of control group (dominant follicle, FD, at withdrawal); 3) at TAI (FD at TAI and vascularization of DF) and 4) seven days after TAI (ovulation rate, area and vascularization of CL). Data were analyzed using Proc GLIMMIX of SAS and the results described as mean  $\pm$  SEM. Short proestrus treated cows had lower (P=0.004) occurrence of estrus (0.0%<sup>b</sup>) than females from control (33.3%<sup>a</sup>) and ECP group (52.9%<sup>a</sup>). There was no effect of treatment (control, ECP and SHORT, respectively) in the any of the other variables: 1) diameter (mm) of DF on eighth day of the treatment (12.0±0.8, 12.1±0.8 and 11.9±0.8, P=0.97), 2) DF at TAI (14.3±0.7, 14.2±0.7, 14.8±0.7, P=0.82), 3) vascularization (%) of DF at TAI (36.1±4.2, 36.8±3.8 and 35.0±4.1, P=0.95), 4) ovulation rate (100.0%, 94.1% and 86.7%, P=0.78), 5) area (mm<sup>2</sup>) of CL (2.9±0.2, 2.9±0.2 and 2.7±0.2 P=0.56), 6) vascularization (%) of CL (63.5±3.3, 65.7±3.1 and 60.4±3.4%, P=0.53) and P4 concentration (4.9±0.4, 4.3±0.4 and 4.2±0.4 ng/ml, P=0.34). It is concluded that the estradiol supplementation or the reduction of proestrus period did not affect the follicular development and the luteal function of cyclic zebu cows synchronized to TAI.

**Acknowledgments:** FAPESP (2012/14731-4).



A114 FTAI, FTET and AI

### **Validation of a method for estrus detection in Nelore females submitted FTAI protocol**

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**Keywords:** bovine, conception rate, estrus.

The study was conducted with the objective to evaluate the efficiency of the chalk marker use as a practical and economical method of estrus detection in Nelore females submitted to the fixed-time artificial insemination (FTAI) program. For that, 410 multiparous Nelore cows with average of postpartum interval of  $64.58 \pm 18.42$  days and body condition score of  $3.19 \pm 0.42$  (1-5 scale) were used, and synchronized with the following protocol: at a random day of the estrous cycle called Day 0 (D0), the animals received an intravaginal progesterone device (P4, PRIMER<sup>®</sup>, Tecnopec, São Paulo, Brazil) associated to 2.0mg of estradiol benzoate (RIC-BE<sup>®</sup>, Tecnopec) intramuscular (im.). On D8, the P4 intravaginal device were removed and 300IU eCG (Novormon<sup>®</sup>, MSD Saúde Animal, São Paulo, Brazil) im., 150µg of d-cloprostenol (Prolise<sup>®</sup>, Tecnopec) im. and 1.0mg of estradiol cypionate (ECP<sup>®</sup>, Pfizer, São Paulo, Brazil) im were administrated. At this time, the animals were marked with chalk maker (RAIDEX, Walmur, Porto Alegre, Brazil) between the sacral tuberosity and the insertion of the tail in a delimited area of 150 cm<sup>2</sup>. The animals remained in the same lot, allowing accept mounting by the females expressed estrus. On D10, the animals were characterized into three groups according to the estrus expression checked by removing chalk maker paint: Group 1- No estrus expression, presence and intensity of color ink (n = 85); Group 2- Intermediate estrus expression, loss of the color intensity of the ink (n = 84) and Group 3- Estrus expression, complete removal of the ink (n = 241). Then, proceeded to measure the diameter of the ovulatory follicle (DFOL) by transrectal ultrasonography using a 5.0 MHz linear transducer (Mindray, DP2200vet, São Paulo, Brazil) and FTAI was performed in all animals. Pregnancy diagnosis was performed by ultrasonography 45 days after FTAI. Data were analyzed by SPSS (version 19). In this study there was an overall mean of DFOL of  $11.81 \pm 3.48$  mm. There was a difference (P=0.0007) among groups 1, 2 and 3 on the DFOL, which showed average of  $10.00 \pm 3.03$ ;  $11.24 \pm 3.46$  and  $12.64 \pm 3.36$  mm, respectively. The overall conception rate was 44.90% (184/410). Groups 1, 2 and 3 had conception rates, respectively, 29.40% (25/85); 33.30% (28/84) and 54.40% (131/241). In comparing the pregnancy rates between groups 1 and 2 was not significant difference; however, group 3 had higher conception rate (P=0.0002) to the other groups. The results of this experiment suggest that estrus detection with the use of the chalk marker is a method of easy applicability, inexpensive and can be used efficiently to assist in the animal identification with high probability of having greater follicular diameter and conception rates.



A115 FTAI, FTET and AI

### **Effect of single-use or re-used intravaginal progesterone devices on the pregnancy rate of lactating *Bos indicus* cows submitted to TAI**

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**Keywords:** fertility, sincronization, Zebu.

The aim of the present study was to evaluate single-use and reused intravaginal progesterone devices on pregnancy rate of lactating *Bos indicus* cows. The hypothesis of the study was that the number of previous use of intravaginal progesterone device or device with single use does not interfere in the pregnancy rate of lactating Nelore cows. In the experiment, 712 lactating *Bos indicus* cows were used with body condition score of  $3.18 \pm 0.01$  (1 to 5 scale). On a random day of the estrous cycle (D0) cows received 2mg of BE (Gonadiol<sup>®</sup>, MSD, Brazil) and were randomly assigned to one of four treatments according to the type of the progesterone device: new DIB group (MSD, Brazil, n = 178), DIB 1x group (previously used for 8 days, n = 184), DIB 2x group (previously used for 16 days, n = 169) or single use group (new device with single use; Cronipres single dose, Biogenesis Bago, Brazil). On D8, all animals received 397.5µg of sodic Cloprostenol (Ciosin<sup>®</sup>, MSD, Brazil), 300IU of eCG (Folligon<sup>®</sup>, MSD, Brazil), 1mg of estradiol Cipionate (ECP<sup>®</sup>, Pfizer, Brazil) and intravaginal progesterone withdraw. Cows were inseminated at fixed time, 48 hours after progesterone device removal. The pregnancy diagnosis was performed 55 days after timed artificial insemination (TAI). The data were analyzed by GLIMMIX procedure of SAS. In these study, there was no difference in pregnancy rate between treatments [new DIB – 56.2% (100/178), DIB 1x – 64.1% (118/184), DIB 2x – 59.2% (100/169) and Single use – 60.8% (110/181); P=0.52]. Therefore, lactating *Bos indicus* cows synchronized with new, reused (previously used for 8 or 16 days) or single use progesterone device had similar pregnancy rates in TAI programs.



A116 FTAI, FTET and AI

### Effect of heat stress and repeat breeding on P/AI of high-producing Holstein cows

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**Keywords:** heat stress, repeat breeder, reproductive efficiency.

This study was conducted on three commercial dairy farms in Carambeí and Céu Azul – PR cities during summer and winter. Heifers (average age of 17.0±0.2 mo and 0.4±0.2 previous services), peak lactation cows (PL; 2.0±0.1 lactations, 103.7±3.2 DIM, average milk production of 39.6±0.6 L/day and 0.9±0.1 previous services) and repeat breeders (RB; 2.1±0.1 lactations, 345.2±10.5 DIM, average milk production of 26.9±0.7 L/day and 5.8±0.2 previous services) started a protocol to synchronize the follicular wave emergence and ovulation for timed artificial insemination (TAI). Thus, 179 heifers, 198 PL and 233 RB cows were treated with a progesterone-releasing intravaginal device (Sincrogest<sup>®</sup>, OuroFino, Brazil) plus 2 mg estradiol benzoate (Sincrodiol<sup>®</sup>, OuroFino) on random days of the estrous cycle (D0). On D7, 530 µg sodium cloprostenol (Sincrocio<sup>®</sup>, OuroFino) was administered. On D8, the progesterone device was removed and 1 mg estradiol cypionate (E.C.P<sup>®</sup>, Pfizer, Brazil) was administered. After 48 hours, all animals were treated with 100 µg gonadorelin (Fertagyl<sup>®</sup>, MSD, Brazil) and TAI was performed. Same batch of a single Holstein bull was used for all TAI. Pregnancy diagnosis was performed 35 days after TAI by ultrasonography. Data were analyzed by logistic regression using the PROC GLIMMIX from SAS. No interaction was found between category (heifer, PL and RB) and season of the year (summer and winter). Overall P/AI of Holstein heifers (43.0%<sup>a</sup>) was greater than PL (25.3%<sup>b</sup>), and both were greater than RB (16.7%<sup>c</sup>; P<0.0001) Holstein cows. Regardless of category, summer heat stress reduced the overall P/AI compared to winter period (21.8% vs 30.8%; P=0.05). When categories were analyzed per season, the following P/AI was observed: heifers during the summer = 37.2% (29/78), heifers during the winter = 49.3% (35/71), PL during the summer = 21.6% (19/88), PL during the winter = 28.2% (31/110), RB during the summer = 11.8% (14/119), and RB during the winter = 21.9% (25/114). In the present study it was observed that RB cows had lesser P/AI compared to heifers and PL cows treated with the same protocol for TAI and using the same batch of semen. This result confirms data found in previous large retrospective studies. Also, the negative impact of heat stress on P/AI was observed in the three categories of Holstein bovine female analyzed herein.

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A117 FTAI, FTET and AI

### **Anti-neospora caninum antibodies occurrence in embryo recipient cows in south western amazon**

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**Keywords:** bovine, embryo, neosporosis.

An animal reproduction disease, neosporosis is widespread in the national herd and to date it has not been diagnosed or reported in Acre State. We used 235 crossbred cows (*Bos taurus* x *Bos indicus*) as embryo recipients, subjected to identical synchronization of ovulation protocol for fixed-time embryo transfer (FTET). On the 16th day after the beginning of the protocol, an *in vitro* produced embryo was transferred to each recipient and, at the same time it was collected from each recipient a blood sample by venous puncture of the coccygeal vein in tubes with vacuum without anticoagulant. The detection of antibodies against *Neospora caninum* was performed by indirect immunofluorescence test and reagent animals were considered with titer  $\geq 200$ . The diagnosis of pregnancy and reassessments of recipients for pregnancy or abortion confirmation were performed on the 25th and 55th days after FTET, respectively, both by ultrasonography (Aloka SSD 550, Aloka, Japan). The immunoassay was performed at the Biological Institute of São Paulo and statistical analysis used was the Chi-square test at a significance level of 5%. Serum-reactivity to *Neospora caninum* was diagnosed in 143 (60.85%) cows. There were 158 (67.23%) recipient cows with CL at D16. Out of the embryo transferred cows, 54 (34.18%) became pregnant and pregnancy loss was 20.37% (11/54). Five (45.45%) cows that had embryo/fetal loss had an indirect immunofluorescence reaction with antibody titers above 400, and one recipient with a 3200 title, which was considered high and suggestive of active infection. The high antibody titers may be associated with a latent infection reactivation, which may have occurred in cows with antibody titer equal to 3200. However the statistical analysis, showed independent variables, i.e., abortion cases were not associated with seropositivity of recipients ( $P>0.05$ ). This is a pioneering report on the occurrence of cow serum reagent to neosporosis in Acre and can serve as a warning to authorities involved in the animal health.



A118 FTAI, FTET and AI

### Effect of numbers of use of a CIDR with 1.9 g or 1.38 g of P4 on pregnancy rate in lactating Nelore cows submitted to a TAI protocol

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**Keywords:** CIDR, pregnancy, progesterone.

The aim of this study was to evaluate the effect from numbers of use of a CIDR with 1.9 g of P4 (CIDR<sup>®</sup>-BR, Zoetis, Sao Paulo, Brazil) or 1.38 g of P4 (CIDR<sup>®</sup>-US; Zoetis, New Jersey, USA) on pregnancy rate in lactating Nelore cows submitted to a synchronization protocol with a new (1<sup>st</sup> use) or a device that had been previously used for 9 (2<sup>nd</sup> use), 18 (3<sup>rd</sup> use) or 27 d (4<sup>th</sup> use). A total of 1,612 Nelore cows with 30 to 50 d postpartum and BCS of 3.02±0.01 (1 to 5 scale) were divided into 15 handling groups. The cows were randomly assigned within handling group to either receive a CIDR<sup>®</sup>-BR or a CIDR<sup>®</sup>-US (1<sup>st</sup> use, 5 groups: BR=317, US=220; 2<sup>nd</sup> use, 2 groups: BR=134, US=131; 3<sup>rd</sup> use, 4 groups: BR=209, US=201 and 4<sup>th</sup> use, 4 groups: BR=211, US=189) and 2 mg im of estradiol benzoate (2.0 mL, Estrogin<sup>®</sup>, Farmavet, Sao Paulo, Brazil) on D0, 12.5 mg im of dinoprost tromethamine (2.5 mL, Lutalyse<sup>®</sup>, Zoetis) on D7, 0.5 mg im of estradiol cypionate (0,25 mL, ECP<sup>®</sup>, Zoetis) + 300 IU of eCG (1.5 mL, Folligon<sup>®</sup>, MSD Animal Health, São Paulo, Brazil) and CIDR<sup>®</sup> withdrawal on D9. All cows were bred by AI 48 h after CIDR<sup>®</sup> withdrawal (D11) and the pregnancy diagnosis were performed by ultrasonography on D41. The PROC GLIMMIX from SAS<sup>®</sup> 9.2 was used to analyze pregnancy rate, were included in the model the effects of AI technician, semen and type of device, significance was set when P<0.05. There was no effect of type of device (P>0.10) when cows were synchronized with a 1<sup>st</sup> (BR: 56.8%; 180/317 vs. US: 63.6%; 140/220), 2<sup>nd</sup> (BR: 55.2%; 74/134 vs. US: 56.5%; 74/131) and 3<sup>rd</sup> use device (BR: 51.2%; 107/209 vs. US: 51.2%; 103/201); however, cows synchronized with a 4<sup>th</sup> use device had the pregnancy rate greater (P<0.05) for CIDR<sup>®</sup>-BR (55.5%; 117/211) in comparison with CIDR<sup>®</sup>-US (41.8%; 79/189). The possible reason for the lower pregnancy with the fourth use from an intravaginal device that contains 1.38 g of P4 could be the lower P4 available during the follicle development.



A119 FTAI, FTET and AI

## Hormone supplementation after artificial insemination increases summer pregnancy rate in dairy cows

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**Keywords:** bovine, GnRH, hCG.

The maintenance of gestation in the cow after artificial insemination (AI) depends upon an adequate embryo development in order to favor the maternal recognition of pregnancy (MRP). The optimization of ovarian luteal function increases circulating progesterone (P4) and contributes to prepare the uterus to receive and nurse the growing conceptus. Therefore, it was devised a hormone strategy to optimize MRP by means of a supplementation based on the combined effects of a GnRH analogue and hCG, given respectively 5 (D5) and 12 days (D12) after AI. The rationale behind the treatment relies on the fact that GnRH on D5 is capable to induce endogenous secretion of LH, ovulate the first wave dominant follicle and generate an accessory corpus luteum (CL), which secretes additional P4 during the MRP. In its turn, hCG on D12 may prevent the presence of a growing DF during the MRP and retard or inhibit PGF<sub>2α</sub> secretion. As a result, luteolysis is delayed, conceptus may grow more during the MRP and pregnancy maintenance is favored. In order to evaluate that protocol, 292 Holstein dairy cows of Embrapa Pecuária Sudeste were artificially inseminated after estrus detection during a two-year long period. Cows were divided into CONT (n=164) - without additional treatment and GnRH/hCG (n=128) - received IM 250 µg of gonadorelin (GnRH) on D5 and 2500 IU of hCG on D12. Pregnancy diagnosis was performed 28 days after AI through transrectal ultrasound (MindRay Vet 3300, 5MHz probe). Pregnancy rate (PR) was analyzed through the Chi-square test ( $\chi^2$ ) using the proc FREQ of SAS statistical package. The PR of cows treated in the autumn/winter was not different between treatments and was 47.9% (34/71) for GnRH/hCG and 47.0% for CONT (47/100). Similarly, overall PR was not statistically different ( $\chi^2=2.05$ ) and was 49.4% (81/164) and 57.8% (74/128) respectively for CONT and GnRH/hCG. However, hormonal treatment resulted in higher PR when the AIs were performed during spring/summer. Indeed, spring/summer PR of GnRH/ hCG was higher (70.2%; 40/57) than ( $P<0.10$ ;  $\chi^2=3.69$ ) PR achieved by CONT (53.1%; 34/64). There was no significant effect of bull or technician on PR in any comparison assessed. In conclusion, the GnRH/hCG combination given after AI increases PR in dairy cows inseminated during spring and summer seasons. It is speculated that luteotrophic action of GnRH/hCG mitigates the effects of environmental stress during early stages of conceptus development.



A120 FTAI, FTET and AI

### **Timed artificial insemination programs during summer in lactating dairy cows: comparison of the 5-d Cosynch protocol with an estrogen/progesterone-based protocol**

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**Keywords:** 5-d Cosynch, dairy cow fertility, E2/P4 protocol.

The objective of this study was to compare a GnRH-based to an estrogen (E2)/progesterone (P4)-based protocol for estrous cycle synchronization and timed artificial insemination (TAI), both designed for synchronization of ovulation and to reduce the period from follicular emergence until ovulation. A total of 1,190 lactating Holstein cows, primiparous (n=685) and multiparous (n=505), yielding 26.5±0.30kg of milk/d were randomly assigned to receive one of the following programs: 5-d Cosynch protocol [D -8: CIDR<sup>®</sup> (Zoetis, São Paulo, Brazil) + GnRH (100mg im gonadorelin, 1.0mL Fertagyl<sup>®</sup>, MSD Animal Health, São Paulo, Brazil), D -3: CIDR removal + PGF<sub>2α</sub> (25mg im dinoprost tromethamine, 5.0mL Lutalyse<sup>®</sup>, Zoetis), D -2: PGF<sub>2α</sub>, D 0: TAI + GnRH]; or E2/P4 protocol [D -10: CIDR + EB (2mg im estradiol benzoate, 2.0mL Estrogin<sup>®</sup>, Farmavet, São Paulo, Brazil), D-3: PGF<sub>2α</sub>, D-2: CIDR removal + ECP (1mg im estradiol cypionate, 0.5mL ECP<sup>®</sup>, Zoetis), D 0: TAI]. Rectal temperature (RT) and circulating P4 concentration were measured on the D -3, -2, 0 and D 7. The estrous cycle was considered to be synchronized when P4≥1.0ng/mL on D 7, in cows that had luteolysis (P4≤0.4ng/mL on D 0). Cows were classified based on the number of times (0, 1, or 2+) they were detected with hyperthermia (RT≥39.1°C). Pregnancy success (P/AI) was determined on D 32 and D 60 after TAI. The binomial variables were analyzed using the PROC GLIMMIX, and the continuous variables using the PROC MIXED of SAS. The cows in the 5-d Cosynch protocol increased (P<0.01) P4 at the time of PGF<sub>2α</sub> (2.66±0.13 vs. 1.66±0.13ng/mL). A greater (P<0.01) number of cows in the E2/P4 protocol was detected in estrus (62.8%) compared to cows in the 5-d Cosynch protocol (43.4%). Occurrence of estrus improved (P<0.01) P/AI in both treatments. Cows in the 5-d Cosynch protocol had greater (P=0.02) percentage of estrous cycle synchronized (78.2%), compared with cows in the E2/P4 protocol (70.7%). On D60, the E2/P4 protocol tended (P=0.07) to improve P/AI (20.7% vs. 16.7%) and reduced (P=0.05) pregnancy loss from 32 to 60d (11.0% vs. 19.6%), compared with 5-d Cosynch protocol. In cows with their estrous cycle synchronized, the E2/P4 protocol had greater (P=0.03) P/AI (25.6% vs. 17.7%) on 60d and lower (P=0.01) pregnancy loss (6.7% vs. 21.7%) compared with cows in the 5-d Cosynch protocol. Follicle diameter affected (P=0.04) pregnancy loss only in the cows in the 5-d Cosynch protocol, with smaller follicles resulting in greater pregnancy loss. P/AI at d60 was different (P=0.01) between protocols in the cows with two or more measurements of heat stress (5-d Cosynch=12.2% vs. E2/P4=22.8%). In conclusion, the 5-d Cosynch protocol produced better estrous cycle synchronization than the E2/P4, however it was observed better P/AI in the E2/P4 protocol.



A121 FTAI, FTET and AI

### **Follicular development in cyclic Nelore cows treated with intravaginal progesterone device used three times or with single-use (single dose).**

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**Keywords:** beef cows, follicular dynamics, intravaginal devices.

Fixed-time artificial insemination programs (FTAI) present significant cost savings when implants of progesterone (P4) are reused (Motlomele et al., 2002, Small Rum. Res., 45, 45-49; Barufi et al., 2002, Rev. Bras. Reprod. Anim., 26, 226-229; Almeida et al., 2006, Braz. J. Vet. Res. Anim. Sci., 43, 456-465). However, when reused, they provide lower plasma P4 concentrations than those observed in the first use. Lower P4 can change the frequency of LH pulses and follicle development pattern with reflections at the wave synchronization, the scattering and ovulation rate and fertility, especially in cows without corpus luteum, which are exclusively dependent of P4 derived from the device. Furthermore, care and cleaning of storage devices are not always observed and often do not guarantee product protection or for who will handle it. To improve the management efficiency and devices safety handling, there is a tendency to use single-use implants. The present study evaluated the differences in the follicular development of cycling cows treated with two different intravaginal P4 devices. We used: (A) 10 cows receiving device with 1 g of P4 (Cronipres of three uses<sup>®</sup>) and (B) 10 cows were given a device containing 0.558 g of P4 (Cronipres Mono Dose<sup>®</sup> M-24). Thus, we performed ovarian ultrasound examinations, every 24 h, from the insertion of the P4 device until its removal, on Day 8. After removal of the P4 device, all cows were examined by ultrasonography every 12 hours for 4 days to determine the size of the follicle at the time of FTAI, ovulation rate and timing of ovulation. We used a completely randomized design with repeated measures. Ovulation data were analyzed by logistic regression using PROC LOGISTIC and follicular development data using PROC MIXED (SAS, version 9.3). The ovulation rate was 80% for both groups ( $P>0.05$ ). There was a tendency to anticipate ovulation ( $P=0.07$ ) in cows from group B. A cow from group B anticipated ovulation in about 36 h. Additionally, group B cows also had a tendency ( $P=0.06$ ) to present a larger diameter of dominant follicles at FTAI. Therefore, from these results, we conclude that cycling cows treated with single-dose P4 device, present pattern of follicular development similar to that of cows treated with devices containing 1 g of P4, however, there is a tendency to anticipate ovulation and to present a bigger preovulatory follicle at the time of FTAI.

**Acknowledgments:** Biogénesis Bagó, Beef cattle Sector in USP Pirassununga.



A122 FTAI, FTET and AI

### **Uterine diameter and induction of ovarian cyclicity in prepubertal Nelore heifers treated with progesterone**

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**Keywords:** *Bos indicus*, heifers, uterus.

The present study aimed to evaluate the effect of progesterone (P4) treatment on the uterine diameter and pregnancy rates of heifers receiving fixed-time artificial insemination (FTAI). A total of 99 Nelore heifers between 22 and 24 months with body condition score between 3.0 and 3.5, in the region of Congoinhas, State of Parana were used. The heifers were submitted to ultrasound examinations of the uterus to measure uterine diameter, after obtaining three transversal evaluations and calculating the average among measurements. The cyclicity was evaluated prior to the hormonal treatment by the presence of the corpus luteum in two ovarian ultrasound examinations, with an interval of ten days. The heifers were separated into two groups according to the presence of the corpus luteum (pubertal heifers, n=36 and prepubertal heifers, n=63). The prepubertal heifers were treated with intravaginal progesterone devices previously used for 32 days (CIDR<sup>®</sup>, Pfizer, Brazil). Ten days later the devices were removed and 1 mg of estradiol benzoate (EB, Estrogin<sup>®</sup>, Farmavet, Brazil) was administered im. Seven days after the EB administration a new ultrasound examination was performed to measure the uterus. The animals received an intravaginal P4 device previously used for 24 days and 2 mg of estradiol benzoate im. On the seventh day 12.5 mg of Dinaprost im (Lutalyse<sup>®</sup>, Pfizer, Brazil) was administered and on the ninth day the device was removed and 400 IU of eCG were administered im (Novormon<sup>®</sup>, MSD, Brazil), and 0.25 mg of estradiol cypionate (ECP<sup>®</sup>, Pfizer, Brazil) im. Forty-eight hours later, the heifers were inseminated with semen from the same bull. The pregnancy diagnosis was performed 35 days after FTAI protocol, by means of ultrasound exam. The results were submitted to analysis of variance ( $P < 0.05$ ). The uterine diameter was different ( $P < 0.05$ ) between the the times before and after treatment with P4 ( $10.0 \pm 1.8$  vs  $12.5 \pm 1.4$  mm) only in prepubertal heifers (pubertal heifers:  $12.0 \pm 1.1$  vs  $12.5 \pm 1.3$  mm). The induction of cyclicity occurred in 71% (44/62) of the prepubertal heifers. The conception rate after FTAI was similar between prepubertal and pubertal heifers (61.9% vs 55.5%, respectively;  $P > 0.05$ ). In conclusion the treatment of prepubertal heifers with P4 was effective in inducing cyclicity, increase uterine diameter and provide adequate pregnancy rates compatible with pubertal heifers.



A123 FTAI, FTET and AI

## Effect of the utilization of diferents intravaginal progesterone device in the fertility of dairy cows

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**Keywords:** CIDR, progesterone, TAI.

The objective of this study was to evaluate the fertility of dairy cows submitted to timed artificial insemination (TAI) protocols using intravaginal device for three times with different progesterone concentration (P4 1.38g x 1.9g). A total of 1,073 lactating Holstein cows (695 multiparous and 378 primiparous) were used in five different farms, with the average of  $173 \pm 4.47$  days in lactation,  $2.42 \pm 0.05$  inseminations,  $32.1 \pm 0.31$  kg of milk/day and  $3.0 \pm 0.02$  of body condition score, in the scale of 1–5. All cows received the following TAI protocol: D0 – 2mg of EB im (2.0mL of Estrogin<sup>®</sup>, Farmavet, SP, Brazil) + intravaginal P4 device (CIDR<sup>®</sup>, Zoetis, SP, Brazil); D7 – 25mg of PGF im (5.0mL de Lutalyse<sup>®</sup>, Zoetis); D8 – removal of the P4 device + 1mg of ECP im (0.5mL, ECP<sup>®</sup>, Zoetis); D10 – TAI. On D0 the cows were randomly assigned to receive CIDR A (1.38g) or B (1.9g), new [1<sup>st</sup> use], previously used for 8 days [2<sup>nd</sup> use] or previously used for 16 days [3<sup>rd</sup> use]. Blood samples were collected on D8 of the protocol to evaluate the P4 concentration. The pregnancy diagnostic was performed by ultrasound 30 days after insemination. Binomial variables were analyzed using PROC GLIMMIX and the continuous by PROC MIXED. Significant differences were considered when  $P \leq 0.05$  and tendency when  $P > 0.05$  and  $P < 0.10$ . The P4 concentration with the CIDR of 1<sup>st</sup> use tended ( $P=0.09$ ) to be higher for CIDR A ( $1.64 \pm 0.10$  ng/mL) compared to CIDR B ( $1.49 \pm 0.10$  ng/mL). There was no difference ( $P=0.36$ ) on the P4 concentration between the CIDR A and B of 2<sup>nd</sup> use ( $A=1.07 \pm 0.7$  ng/mL vs.  $B=1.13 \pm 0.7$  ng/mL). In the 3<sup>rd</sup> use, the CIDR A tended ( $P=0.10$ ) to present lower P4 concentration ( $0.95 \pm 0.13$  ng/mL), when compared to CIDR B ( $1.12 \pm 0.13$  ng/mL). There was no effect ( $P=0.68$ ) on P/AI between CIDR A and B of 1<sup>st</sup> use [ $A=25.2\%$  (48/190) vs.  $B=27.1\%$  (51/188)] and of 2<sup>nd</sup> use [ $A=25.8\%$  (48/182) vs.  $B=24.8\%$  (46/182);  $P=0.82$ ]. Effects ( $P=0.02$ ) were observed between the type of CIDR on P/AI with CIDR of 3<sup>rd</sup> use [ $A=21.6\%$  (37/171) vs.  $B=33.1\%$  (53/160)]. Cows with P4 concentration on the D8 lower than 0.5 ng/mL [10.6% (114/1073)] had lower ( $P=0.01$ ) P/AI [16.6% (19/114)] compared with  $\geq 0.5$  ng/mL [27.4% (264/959)]. There was no effect ( $P=0.99$ ) on the distribution of cows with  $P4 < 0.5$  ng/mL on D8 by the type of CIDR ( $A=1\%$  vs.  $B=1\%$ ) of 1<sup>st</sup> use. There were differences on the distribution of cows with  $P4 < 0.5$  ng/mL at D8 between the type of CIDR when used for the 2<sup>nd</sup> ( $A=12\%$  vs.  $B=3\%$ ;  $P < 0.01$ ) and 3<sup>rd</sup> time ( $A=32\%$  vs.  $B=16\%$ ;  $P < 0.01$ ). Cows with temperature  $\geq 39.1^\circ\text{C}$  at the moment of AI had lower ( $P=0.04$ ) P/AI [21.3% (36/169)], compared to cows with temperature  $< 39.1^\circ\text{C}$  [29.8% (117/393)]. Both intravaginal P4 devices had similar pregnancy results when used for two times, however, when used for more than 16 days, the CIDR with 1.38g of P4 resulted in lower P/AI.



A124 OPU-IVP and ET

## Conception and calving rates and pregnancy loss of vitrified embryos produced *in vitro* of Nelore cows under different diets

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**Keywords:** conception, embryo, Nutrition.

The aim of this study was to evaluate the influence of high or low dry matter intake (DMI) and/or energy on conception rate, calving rate and pregnancy loss of vitrified embryos produced *in vitro*. Non-lactating Nelore cows (N=33), aged between 4 and 10 years, mean weight of 489.5±11.3 kg and BCS 3.25 (scale from 1 to 5) were used. Cows were confined without access to pasture, with two animals per stall. Mineral salt was provided in the diet and water *ad libitum*. After 15 days on the adaptation diet, cows were blocked by initial weight and randomly allocated in four experimental groups. The maintenance group (M) received a diet of weight maintenance consuming 1.2% of DM per kg of body weight (BW). The restriction group (0.7M) received the equivalent of 70% of the group M diet consuming 0.84% of DM per kg of BW. High intake group (1.5M) received the equivalent of 150% of the M group. The energy group (E) received a diet with DM similar to M group, however, with energy level equivalent to 1.5M (TDN = 75%). The cows received all the diets in a latin-square design. There were four sessions of ovum pick-up (OPU), 30 days apart. The oocytes were classified and taken to *In Vitro* Brazil laboratory, where all procedures of IVP and vitrification were realized. Embryos were thawed, evaluated and transferred to synchronized recipients [D0: insert of progesterone (P4) device and 2 mg estradiol benzoate (EB); D8: 0.6 mg estradiol cypionate (ECP), 0.5 mg sodium cloprostenol (PGF2 $\alpha$ ) and 300 IU equine chorionic gonadotropin (eCG); D16 and D17: embryo transfer]. The embryos were transferred by two experienced technicians. A total of 543 embryos were transferred to recipients with CL. Pregnancy diagnosis was performed by US 23 and 53 days after embryo transfer. All data of oocyte and embryo production have been described by Prata *et al.* (2011, *Acta Sci Vet*, v.39:1, p.338). Data were analyzed by PROC GLIMMIX of SAS and the results are presented as least squares means  $\pm$  SEM following the order of treatments M, 0.7M, 1.5M and E. There was no difference in conception rate (%) among treatments at 30 (30.7 $\pm$ 6.8; 38.7 $\pm$ 6.8; 31.3 $\pm$ 6.8 and 34.7 $\pm$ 6.6;  $P=0.49$ ) and 60 (24.6 $\pm$ 5.6; 33.5 $\pm$ 5.5; 25.6 $\pm$ 5.7 and 29.2 $\pm$ 5.4;  $P=0.43$ ) days of pregnancy. Calving rate (%) was also similar among groups (21.1 $\pm$ 3.9; 26.4 $\pm$ 3.4; 22.5 $\pm$ 3.3 and 25.7 $\pm$ 3.5;  $P=0.73$ ). Pregnancy loss (%) between 30 and 60 days (20.0 $\pm$ 6.3; 13.3 $\pm$ 4.8; 18.0 $\pm$ 5.2 and 16.0 $\pm$ 5.2;  $P=0.84$ ) and between 30 days and calving (31.4 $\pm$ 3.2; 31.6 $\pm$ 3.2; 28.0 $\pm$ 3.0 and 26.0 $\pm$ 3.3;  $P=0.89$ ) did not differ among groups. Therefore, changes in DMI or energy for a 30 day-period in non-lactating Nelore cows did not influence the conception rates of their vitrified IVP embryos.

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A125 OPU-IVP and ET

## Effect of oocyte density on commercial *in vitro* bovine embryo production

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**Keywords:** cleavage rate, efficiency, embryo production rate.

The efficiency of the *in vitro* bovine embryo production (IVEP) is the key to increase the profitability of commercial labs. Some factors may affect embryo development *in vitro*, such as oocyte quality, energy substrate, oxygen tension, and embryo density (Khurana et al., *Theriogenology*, 54, 741-766). In ovum pick up (OPU) procedures the number of the oocytes recovered can be variable and low and, in commercial labs, oocytes and embryos have to be cultured from each individual donor cow, regardless of the recovered number, becoming necessary to process them in small groups. It has been reported that optimum development is achieved when oocytes are cultured in groups of 20-40, in comparison with groups of 5 and 10 structures (O'Doherty et al., *Theriogenology*, 48, 161-169). Therefore, the aim of this study was to investigate the effect of oocyte density on IVEP efficiency, which was conducted in a commercial lab of *in vitro* fertilization (IVF). In the lab, cumulus-oocyte complexes (COCs), obtained from random donors, were assigned in six groups: 1-5 COCs per drop (group 1, n=37 drops), 6-10 COCs (group 2, n=91), 11-15 COCs (group 3, n=89), 16-20 COCs (group 4, n=57), 21-25 (group 5, n=45) e 26-30 COCs (group 6, n=27), where n is the number of replicates of each group. Oocyte maturation was performed for 24 hours in drops of 80 µL. After that period, COCs were fertilized with semen from different bulls, in drops of 70 µL for 22 hours. After IVF, presumptive zygotes were cultured for seven days, in drops of 60µL. The percentage of cleavage (third day of culture) and blastocysts (seventh day of culture) were calculated on the number of viable oocytes. All the steps of embryo production were performed with the same conditions in the six groups, including volume of media and the incubation at 38,8 °C in a 5% CO<sub>2</sub> in air with high humidity. Data were analyzed by the method of least squares using analysis of variance by proc GLM. Differences between means were compared by Tukey test with 5% significance. Differences in cleavage rate between groups were not observed (groups 1-6, respectively: 84.9%±32.6%, 82.5%±26.1%, 85.3±19.5%, 80.3%±22.4%, 77.6%±22.5% and 82.1%±21%, P>0.05). Embryo production rate also did not differ between groups (groups 1-6, respectively: 35.4%±42.9%, 35.5%±37.8%, 40.5%±31%, 28.7%±26.3%, 30.5%±25.2% and 39.4%±28.6%, P>0.05). In conclusion, there was no influence of oocyte density on the IVEP, which confirms the efficiency observed in commercial scale, in which the number of oocytes is variable. Besides, other factors - as donors and bulls - which were not the focus of analysis in this study, can be involved.



A126 OPU-IVP and ET

### **Reduction in superovulation response of female bovine superstimulated with FSH in split dose**

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**Keywords:** CL, FSH, superovulation.

This experiment aimed to evaluate the superovulation of Gyr (zebu) females, using conventional protocol with 8 decreasing doses with an interval of 12 hours or protocol with fewer applications and similar dosage (split dose). Sixteen females (total of 32 superovulations), aging 17-42 months, with body condition score varying from 2,5 to 4 (scale 1-5) were used, in a cross-over design. At the beginning of the treatment (D0), animals received progesterone device (Primer® - Tecnopec, Brazil) plus 2 ml of estradiol benzoate (Ric-Be® - Tecnopec). The females of the conventional group received 250 IU of FSH / LH (Pluset® - Hertape Calier - Brazil) divided into eight decreasing doses administered in 12-hour interval (FSH / LH in D4, D5, D6 and D7 in the morning and afternoon, with their respective strengths: 50 IU; 37.5 IU; 25 IU and 12.5 IU). On D7 (morning), females were treated with 2 ml of cloprostenol (Veteglan® - Hertape Calier), and removal of progesterone device was done in the afternoon of D7. The females of the split group also received 250 IU FSH / LH. On D4, 62.5 IU FSH/ LH intramuscular and 125,0 IU subcutaneously were administered in the morning. Twenty-four hours later, 62.5 IU were administered subcutaneously in the morning, and on D7 progesterone device was removed and 2 ml of cloprostenol were administered. Females of both groups received 2 ml of GnRH (Gestran® - Tecnopec) in the morning of D8, and they were inseminated 12 and 24 hours later. On D15 embryo recovery was performed in both treatments. Superovulation response was done counting the number of corpora lutea in each ovary, with the aid of ultrasound. There was a reduction in the number of CLs at the time of collection ( $8.12 \pm 3.26$  and  $4.69 \pm 3.46$ ).



A127 OPU-IVP and ET

## Use of the SPOM system for maturation of bovine oocytes used for IVP

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**Keywords:** oocyte competence, maturation, meiotic arrest.

One of the limitations of the assisted reproductive techniques (ATRs) is the availability of competent oocytes. Therefore, the development of alternatives to increase the competence of oocytes used in ATRs (Hyttel et al., 1997, *Theriogenology*, 47, 23-32) is needed. This study aimed to test the SPOM system (*Simulated physiological oocyte maturation*; Albuz et al., 2010, *Human Reproduction*, 25, 2999-3011) in IVM of bovine oocytes by changing the FSH source, gaseous atmosphere and protein supplement. SPOM involves two steps the first is a short culture period (2h) in the presence of cAMP modulators agents (forskolin 100  $\mu$ M, 500  $\mu$ M of IBMX, Sigma Sto. Louis, USA) and the second is an extended IVM (30h) in the presence of 20 $\mu$ M of cilostamide (Sigma Sto. Louis, USA), associated with a high concentration of FSH (0.1 IU / ml). COCs (n = 452) were aspirated from follicles of 3-8 mm and distributed into four groups: G1: control, oocytes submitted to IVM, IVF and IVC, previously described (Machado et al., 2012, *Zygote*, 20, 123 - 134); G2: SPOM FSHr +BSA, oocytes submitted to the SPOM with recombinant FSH (FSHr) and 4 mg/ml FAF-BSA, 5% O<sub>2</sub> during IVM; G3: SPOM with FSH + BSA, oocytes submitted to the SPOM with porcine FSH (FSHs) and 4 mg/ml FAF-BSA, 5% O<sub>2</sub>; G4: SPOM with FSH+10% FCS, oocytes submitted to the SPOM with FSHs and 10% FCS in atmosphere of 5% CO<sub>2</sub> in air; G5: SPOM FSHr + 10% FCS, oocytes submitted to the SPOM with FSHr and 10% FCS in 5% CO<sub>2</sub> in air. After IVM, oocytes were *in vitro* fertilized and cultured until D8. At that time, blastocysts were measured and those greater than 160  $\mu$ m were stained with Hoechst

33342 to assess total cell number. Data of embryonic development were analyzed using  $\chi^2$  test (P  $\square$  0.05) and

embryo size and total cell number were compared using the Kruskal-Wallis test (P  $\square$  0.05). Cleavage rate was similar (P > 0.05) between G1 (77.5%), G2 (70.3%) and G3 (67.4%), but lower than (P < 0.05) G4 ( 54.5%) and G5 (37.7%). Blastocyst rate differed (P < 0.05) among all groups being higher in G1 (42%), intermediate in G2 (26.5%), G3 (33.3%) and G4 (12.0%) and lower in the G5 (5.7%). When SPOM was performed under low O<sub>2</sub> tension, the size and number of cells in G2 and G3 embryos were similar (P > 0.05) to G1. In G4 and G5, the size of embryos (163.6  $\pm$  63.9 and 171.9  $\pm$  41, respectively) and the number of cells (125  $\pm$  41 and 96  $\pm$  7.7, respectively) were lower than the other groups. Results suggest that SPOM system, regardless of the FSH source and O<sub>2</sub> tension, did not increase blastocyst production neither affect their quality. However, SPOM in the presence of FCS and high O<sub>2</sub> tension in IVM, in spite of FSH used, have a deleterious effect on *in vitro* embryo production.



A128 OPU-IVP and ET

### **Effect of bull in in vitro embryo production of Holstein cows with sexed or conventional semen**

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**Keywords:** IVP, OPU, sexed-semen.

The aim of this study was to evaluate the bull effect and the type of semen (sexed or conventional) used in in vitro embryo production from Holstein females. In the study, 660 Holstein oocytes donors of five different commercial farms were used during 2010 and 2011. A total of 1,814 ovum pick-up (OPU) sessions were performed on random day of the estrous cycle. All laboratory procedures, in vitro maturation, fertilization and culture were performed by the same method and laboratory (In Vitro Brasil, Mogi Mirim, SP, Brazil). For in vitro fertilization (IVF) 40 bulls were used: 28 had sexed semen and 12 had conventional semen. The variable analyzed was embryo production rate by OPU session (number of viable embryos per number of viable oocytes). Data were analyzed using the GLIMMIX procedure of SAS. The embryo production rate by OPU session was lower when sexed semen was used for IVF [24.5% (3,117/12,739)], compared with the conventional semen [35.1% (1,162/3,370),  $P < 0.0001$ ]. In contrast, after selecting 1/3 of bulls with the highest in vitro embryo production performance with sexed semen ( $n=4$ ) and conventional semen ( $n=9$ ), similar embryo production rate was observed [43.9% (645/1,468)], compared with conventional semen [39.8% (454/1,140);  $P = 0.06$ ]. In conclusion, in vitro embryo production is reduced when sexed semen is used. However, it is possible to achieve similar rate between conventional and sexed semen when bulls with higher performance for in vitro embryo production is used. The data indicate that there is a significant effect of bull on in vitro embryos production when sexed semen is used.



A129 OPU-IVP and ET

### The overstimulation treatments using Folltropin or Pluset showed similar efficiencies in non-lactating Holstein donors

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**Keywords:** dairy herd, *In vivo* embryo, superovulation.

The present study aimed to evaluate the superovulatory response and *in vivo* embryo production in non-lactating Holstein donors treated with Folltropin (Tecnopec, Brazil) or Pluset (Hertape Calier, Brazil) protocols. The study was performed in a commercial dairy farm (Agrindus S/A, Descalvado - SP). A total of 31 donors were initially allocated into two experimental groups in a cross-over design, totalizing 62 superovulation protocols (SOV). Females received two different superovulation protocols using either Folltropin (FOLL; n=31) or Pluset (PLUS; n=31). At a random day of the estrous cycle (D0; AM), cows received an intravaginal progesterone device (P4; Primer, Tecnopec) and 2.0 mg intramuscular (IM) of estradiol benzoate (RIC-BE, Tecnopec). On D4, cows from FOLL group received 300 mg Folltropin diluted into 18 ml of saline solution and injected in 8 decreasing doses (4 ml, 4 ml, 3 ml, 3 ml, 2 ml and 2 ml), 12 h apart. Cows from PLUS group received 400 IU of Pluset<sup>®</sup> diluted in 18 ml of saline and injected in 6 decreasing doses (4 ml, 4 ml, 3 ml, 3 ml, 2 ml and 2 ml), 12 h apart. On D6 (AM and PM) two doses of 0.150 mg of cloprostenol (Estron, Tecnopec) were injected. Furthermore, all animals received 400 IU of eCG (Folligon, Intervet, Brazil) at D7 AM. All P4 devices were removed on D7 PM and 62.5 µg IM of lecorelin (Gestran, Tecnopec) were injected 12 h later (D8 AM). The females underwent timed artificial insemination on D8 PM and D9 AM and embryo recovery performed on D15. Immediately before uterine flushing, the number of corpus luteum (CL) was evaluated and recorded. The same sire was used in both superovulation protocols for each female. Statistical analysis was performed using the GLIMMIX procedures of SAS. There was no difference between experimental groups for superovulation rate (FOLL: 96.8% vs. PLUS 90.3%; P = 0.33), total number of recovered ova (FOLL: 10.4±1.5 vs. PLUS: 7.7±1.6; P=0.08) and number of viable embryos (FOLL: 3.2±0.7 vs. PLUS: 3.6±0.8; P=0.59). However, there were differences on the total of CL (FOLL: 13.2±1.8 vs. PLUS: 10.6±1.8, P=0.04), recovery rate (FOLL 78.6%; 311/396 vs. PLUS: 70.9%; 216/305; P=0.04) and unfertilized ova (FOLL: 6.0±1.4 vs. PLUS: 3.2±1.0; P=0.04). In conclusion, although the FOLL group presented highest superovulatory response, the recovery of viable embryos per flushing was similar to between treatments. Therefore, the superstimulatory treatments (300 mg of Folltropin and 400 IU of Pluset) showed similar efficiency in SOV program of non-lactating Holstein donors.

**Acknowledgments:** Agrindus S.A and Tecnopec.



A130 OPU-IVP and ET

### **The oxygen tension and oocyte density utilized on IVM affects *in vitro* fertilization rates of bovine oocytes**

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**Keywords:** IVF, oocyte density, oxygen tension.

The *in vitro* maturation (IVM) of bovine oocytes, have an important role in the success of *in vitro* fertilization (IVF). Many factors can influence the IVM events, including oxygen tension and oocyte density by volume of medium. Inadequate combination of these factors can induce ROS generation, affecting the fertilization and embryo development. The aim of this study was to evaluate the influence of the association of oxygen tension (5% or 20%) with different oocyte density by volume of medium (1:10 or 1:20 $\mu$ L) on the rates of *in vitro* fertilization in cattle. Bovine oocytes (n=331) were obtained from slaughterhouse ovaries, and after selection, they were randomly allocated into 4 treatments: T1: 1:10 in 5% of O<sub>2</sub>; T2: 1:10 in 20% of O<sub>2</sub>; T3: 1:20 in 5% of O<sub>2</sub>; T4 1:20 in 20% of O<sub>2</sub>. The oocytes were matured in TCM 199 plus 10% of estrous mare serum, EGF, LH, FSH and piruvate for 22 to 24 h in 39°C and saturated humidity. For IVF, the spermatozoa were sorted by Percoll gradients (90, 60, 30%), and co-incubated with oocytes for 18 h in 5% of CO<sub>2</sub> in air and saturated humidity, in FERT TALP medium. Before IVF, cumulus cells were removed by successive pipetting. The presumptive zygotes were stained with Hoescht 33342 and the fertilization rates were evaluated by pro nucleus formation and sperm penetration. The statistical analyses were performed by Z test, with 5% of significance. The rate of normal fertilization (NF) of group T2 (35.37%) was higher than T1 (18.18%; P<0.05) but similar to T3 (26.83%; P>0.05) and T4 (21.52%; P>0.05). The rate of normal penetration (NP) rates did not differ between treatments (P>0.05). When the rates of NF and NP were analyzed in association, the T2 (48.78%) was higher than T1 (29.55%; P<0.05) and T4 (29.11%; P<0.05), but similar to T3 (40.24%; P>0.05). These data show that the system utilized on T2 (1:10 in 20% O<sub>2</sub>) and T3 (1:20 in 5% of O<sub>2</sub>) presented the best rates of IVF, suggesting that when an higher oxygen tension (20%) is used the oocyte density must be high (1:10). A lower oocyte density (1:20) requires a lower oxygen tension (5%). The rates of IVF are influenced by oxygen tension and oocyte density by volume of medium utilized on IVM.

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### Differences in *in vitro* fertilizing capacity and its relation with oxidative stress in cattle

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**Keywords:** IVF, oxidative stress, sperm selection.

The variation among bulls in sperm characteristics, fertilization and embryonic development rates, as long as some factors that induce infertility in males, such as oxidative stress, are obstacles to commercial IVF (Silva *et al.*, Theriogenology, v.67, p.609-619, 2007). This study aimed to determine the effect of the oxidative stress in sperm morphofunctional characteristics, *in vitro* fertilizing capacity and subsequent embryonic development of semen from different bulls. In 5 replicates semen samples from four *Bos taurus* bulls were thawed for 30" in a 35°C water bath. The sperm was sorted by Percoll gradients (Folchini *et al.*, Rev. Bras. Reprod. Anim., v.36, p.239-244, 2012). After that the samples were evaluated for vigor, motility, concentration, morphology, production of reactive oxygen species (ROS), membrane integrity (HOS+), lipid peroxidation, glutathione (GSH) levels and superoxide dismutase (SOD) activity. A dose of  $2 \times 10^6$  spermatozoa/mL of each bull was used to IVF. Presumptive zygotes (100/treatment) were denuded and incubated with Hoechst 33342 solution (10mg/mL) and evaluated for the presence of spermatozoa penetrated, formation of the pronuclei or nuclei fused. Some of the presumptive zygotes (10/treatment) were individually cultured for 48 h in SOFaaci + 10% ESS and BSA in petri dishes over an embryonic monitoring system (Primo Vision, Cryo Management Ltd., Hungary). Embryonic development was assessed by the cleavage rate, time of first cleavage, and average number of blastomeres at 48h. Data were analyzed by chi-square ( $X^2$ ) and ANOVA and the means were compared by Tukey test at 5%. No difference was observed in morphofunctional characteristics. Bulls 2 and 4 ( $49.20 \pm 7.20$  and  $54.80 \pm 7.89$ , respectively) in relation to bulls 1 and 3 ( $35.20 \pm 7.02$  and  $37.20 \pm 6.62$ ). ROS levels were increased for bull sperm 4 ( $76.01 \pm 4.43$ ) when compared to bull 1, bull 2 and bull 3 ( $55.28 \pm 11.54$ ,  $63.78 \pm 8.54$  and  $59.68 \pm 12.22$ ). GSH levels were reduced in bulls' sperm 2 and 4 ( $27.27 \pm 3.82$  and  $27.97 \pm 1.03$ ) when compared to bulls 1 and 3 ( $31.77 \pm 2.74$  and  $33.45 \pm 2.75$ ). SOD activity was increased in bull 1 ( $9.72 \pm 2.50$ ) in relation to bulls 2, 3 and 4 ( $6.79 \pm 1.12$ ,  $6.58 \pm 1.64$  and  $7.45 \pm 1.35$ ). There was no difference in the lipid peroxidation levels, cleavage rate and time of the first cleavage. The bull 1 showed an increased SOD activity and had lower cells number after 48 h (2.5) and total penetration and fertilization rates (63.0%) compared to the others (80.8, and 88.0%, respectively). The bull 4, which showed a high level of ROS, had higher penetration and normal fertilization rates (80.0%) and number of embryonic cells at 48 h after IVF suggesting that ROS showed a beneficial effect. These results show that there are variations in the fertilizing capacity between bulls related to oxidative stress.



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### **Pregnancy after transfer of donkey embryo in cycling mule as recipient**

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**Keywords:** embryo transfer, mule, recipient.

Mules (*E. asinus* X *Equus caballus*) are usually infertile animals. However, some mules have regular cycles and could be considered as recipients, especially for donkey embryos, since this kind of embryo usually has problems when transferred to mares. When using mares as recipients the embryo losses are high, due to the non-invasion of trophoblast cells in the endometrium, with insufficient formation of endometrial cups and no production of eCG. The aim of this report was to evaluate the feasibility of using a mule as an alternative recipient to donkey embryo. The control of the follicular wave was performed after detection of estrus of the donkey donor. When the donor presented a pre-ovulatory follicle of 38 mm and uterine edema grade 3 (scale 1-3), the ovulation was induced with hCG (2500UI) IM. One day later, the donor was submitted to the copulation with a male donkey of proven fertility and detection of ovulation occurred within 48 hours. At D8.5 post-ovulation the embryo collection was performed by non surgical method in dual system with approximately 1 L of ringer's lactate. After three flushes, the content was recovered in petri dishes for the embryo detection. After identification, the embryo was washed in 10 drops of Holding plus (Vitrocell / Embriolife ®) and transferred to the recipient mule. This recipient showed signs of estrus two days after the donor. After checking the presence of a follicle of 37 mm and uterine edema grade 3, the ovulation was induced with hCG (2500 UI) IM and the ovulation was detected by ultrasonography. On the day of transfer (D5), the mule had firm uterine tonus and corpus luteum of approximately 30 mm. The transfer of the embryo into the uterine body required also transrectal palpation because of the difficulty in passing the small cervix. After five days, the pregnancy diagnosis was done and it was confirmed at 60 days post-transfer, both by transrectal ultrasonography. We conclude that it is possible to use natural cycles of cycling mules as a viable alternative to achieving better pregnancy rates of donkey embryos, even without the use of exogenous progesterone to maintain pregnancy.



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### Effect of hormonal treatments pre-OPU on oocyte recovery and *in vitro* embryo production in Girolando cows (*Bos taurus* x *Bos indicus*)

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**Keywords:** dairy cows, OPU, synchronozation – FSH.

The Girolando (*Bos taurus* x *Bos indicus*) breed is widespread in Brazil due to its morphologic and physiologic characteristics being favorable for dairy production in the tropics. The success and efficiency of the *in vitro* embryo production mainly depend upon the quantity and quality of oocytes recovered in OPU. To enhance the efficiency of the technique, the use of hormonal treatments pre-OPU may be necessary. The objective of this study was to compare oocyte recovery in the presence of a CL or not at the moment of the OPU and blastocyst production of cows subjected to different hormonal protocols pre-OPU. Twelve cyclic and non-lactating Girolando cows were blocked by parity and genetic traits and were randomly assigned to three groups: G1 - OPU in a random day of the estrous cycle; G2 - follicular wave synchronization, in which cows received on D 0 an intravaginal device of progesterone (CIDR<sup>®</sup>, Zoetis, Auckland, New Zeland), 2 ml of estradiol benzoate I.M. (Gonadiol<sup>®</sup>, Schering-Plough, Sao Paulo, Brazil) and 2 ml of PGF<sub>2α</sub> I.M. (Ciosin<sup>®</sup>, Schering-Plough, New Jersey, EUA), and on D 5 OPU was performed; G3 - similar to G2, adding I.M. injection of 40 mg of FSH (Folltropin<sup>®</sup>, Bioniche, Belleville, Canada) on D 3. The cows underwent a total of six OPUs in a cross-over design, in which all of them went through all the treatments twice, in 30 days apart (between one OPU session and the next one). During OPU, the number of aspirated follicles was recorded and the presence of a CL was verified. Oocytes retrieved were quantified and classified, as well as the number of blastocysts produced in each experimental group. Data were analyzed by ANOVA. In a total of 68 OPU sessions, 778 follicles were aspirated, resulting in 689 COCs recovered (88.6%; G1 - 260; G2 - 278; G3 - 240; P>0.05). The average of aspirated follicles, oocytes recovered and viable oocytes per experimental group were, respectively: G1 - 10.8 / 9.4 / 6.1; G2 - 12.1 / 11.8 / 8.0; G3 - 11.4 / 9.1 / 6.2; (P>0.05). The average of *in vitro* matured COC, number of cleaved embryos and blastocysts produced per treatment were, respectively: G1 - 6.9 / 5.7 / 1.3; G2 - 6.8 / 5.2 / 1.3; G3 - 6.5 / 6.0 / 1.8 (P>0.05). The number of CL present at OPU was greater (P<0.05) in G1 (16 in 24 OPU) than in synchronized groups (G2 - 5 in 23 OPU; and G3 - 5 in 21 OPU). Hormonal protocols of follicular wave synchronization were effective to regress the CL, which can facilitate the OPU procedure. The FSH dose used did not influence the quantity and quality of oocytes recovered. Also, hormonal treatments to synchronize the follicular wave and the use of low dose of FSH aiming the efficiency of OPU did not improve embryo production. In summary, in Girolando breed, OPU performed every 30 days results in similar embryo production between synchronized and FSH-stimulated cows and those without a treatment previously to OPU.



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### The FSH stimulus prior to the ovum pick-up increases the success of *in vitro* embryo production programs in Holstein cows

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**Keywords:** dairy herd, *in vitro* embryo, superstimulation.

The study evaluated the effect of FSH treatment (Folltropin, Tecnopec) prior the Ovum Pick-Up (OPU) using the slow release diluent MAP5 (hyaluronic acid, Bioniche) on *in vitro* embryo production (IVP) program of lactating and non-lactating Holstein cows. A total of 30 cows were used (n=15 lactating and 15 non-lactating cows) in a *cross-over* experimental design, from Agrindus S/A dairy farm, Descalvado-SP. The females were randomly allocated into three groups: control group (CON; n=30); Folltropin 200 mg (FOLL; n=30); MAP5/Folltropin 200 mg (MAP/FOLL, n=30). At a random day of the estrous cycle (D0 AM) all cows received an intravaginal progesterone device (P4; Primer, Tecnopec) and 2.0 mg of estradiol benzoate (RIC-BE, Tecnopec), intramuscular (IM). On D4 and D5 (AM and PM), the FOLL group received four decreasing doses of FSH IM (D4 AM and PM = 4ml; D5 AM and PM = 3 ml). The MAP5/FOLL group received a single dose of 5 ml IM of MAP5 with Folltropin on D4 AM. The P4 devices were withdrawal on D7 AM and cows were submitted to OPU at same day. Immediately before the OPU, all visible follicles were quantified and classified according to their diameter [small (SF = <6mm), medium (MF = 6 to 10mm) and large (LF = >10 mm) follicles. The same mating was maintained for all IVP procedures during the experiment. Variables were analyzed by the GLIMMIX procedure of SAS. There was no difference among experimental groups on the number of follicles aspirated (CON: 17.1 ± 1.1; FOLL: 17.2 ± 1.3; MAP5/FOLL: 18.3 ± 1.5; P = 0.34), total oocytes recovered (CON: 12.0 ± 1.2; FOLL: 10.3 ± 1.0; MAP5/FOLL: 10.9 ± 1.3; P = 0.21), number of LF (CON: 2.1 ± 0.2; FOLL: 1.5 ± 0.3; MAP5/FOLL: 1.8 ± 0.3; P = 0.06) and viable oocytes (CON: 9.3 ± 1.0; FOLL: 8.6 ± 0.9; MAP5/FOLL: 8.7 ± 1.2; P = 0.73). However, cows from FOLL (9.5 ± 1.1) and MAP5/FOLL group (8.9 ± 1.2) had higher number of MF compared to cows from CON group (3.61 ± 0.6, P < 0.0001). In contrast, fewer number SF was observed in FOLL (6.2 ± 1.0) and MAP5/FOLL (7.7 ± 1.0) compared to the CON (11.5 ± 1.0<sup>a</sup>, P < 0.0001). It was also observed lower recovery rate in the FOLL (60%; 310/517) and MAP5/FOLL (59.5%; 327/550) compared to the CON (69, 8%; 359/514; P < 0.0001). However, the FOLL (34.5%; 89/258) and MAP5/FOLL (25.3%; 70/277) groups had higher blastocyst rate than the CON group (19.8%; 55/278<sup>b</sup>; P < 0.0009). The FOLL (3.0 ± 0.5) resulted in higher number (P = 0.03) of viable embryos per OPU session compared to the CON (1.8±0.4<sup>b</sup>) and an intermediate value was obtained in MAP5/FOLL group (2.3 ± 0.5<sup>ab</sup>). In conclusion, FSH treatment prior to the OPU, with or without MAP5, increased the success of IVP programs in Holstein cows.



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## Results of OPU-IVP of *Bos taurus* donors over the year in subtropical climate

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**Keywords:** bovine, embryos, oocytes.

*Bos taurus taurus* donors besides having lower efficiency in the process of genetic multiplication by *in vitro* embryo production (IVEP) may also changes in their results according to the weather, especially in tropical regions (Al-Katanani et al. 2002, J. Dairy Sci., 85, 396-403). This study aimed to compare the distribution of IVEP results over the months of the year in donors *Bos taurus taurus*, done in the south and southwest of the Minas Gerais state (Brazil) by the same veterinarian. The region has mesothemic climate of CW classification, according to Koepen. Results from 960 follicular aspirations (OPU) in Holsteins (476), Simental (264), Angus (142) e Jersey (78) donors, done for a period of 12 months, were used. In this period the total rainfall was 2.970 mm, the maximum temperature was 38.7°C and the minimum was 4.6°C. The management of the animals was semi-confinement with supplementation of corn silage and commercial grain concentrated. For OPU, an ultrasound device with intravaginal microconvex transducer of 7,5 MHz (Mindray DP 2200) was used. All follicles larger than 3 mm were identified and punctured. The cumulus oocytes complexes (COCs) recovered were counted and classified based on their morphological aspect. The viable oocytes were matured *in vitro* (TCM 199) for 22-24 hours after the start of OPU at a temperature of 38.5°C, 5% CO<sub>2</sub> and saturated humidity. Sorted sexed semen from different bulls, evaluated for motility and vigor, was used for *in vitro* fertilization (IVF). After IVF the presumable zygotes were transferred to *in vitro* culture (SOF), where they remained for seven days at a temperature of 38.5°C and controlled atmosphere (5% CO<sub>2</sub>). The average production of oocytes and embryos conversion was compared between breeds and months of the year by Dunn's test, considering a 5% significance. There was no breed effect for any variable. The donors produced an average of 11.6±9.8 total oocytes, 6.9±6.4 viable oocytes, and 1.6±0.9 embryos per OPU. Unlike described in other studies (Edwards *et al*, 1996, Biol. Reprod.,55, 341-346; Camargo *et al*, 2007, Theriogenology, 68, 626-632) no differences were observed in the total production of oocytes and embryos in different months of the year. The conversion rates of oocytes to embryos was also similar in different months of the year (P>0.05). It is believed that the difference compared to other studies is due to management conditions that can be very different between breeding systems. We conclude that, in the studied region, *Bos taurus taurus* donors did not demonstrate effects of seasonal climate variation in the production of viable and total oocytes, as well in the conversion of oocytes to embryos.

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### The use of FSH diluted with slow release carrier (MAP5) reduces the efficiency of *in vivo* embryo production protocols of Holstein donors

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**Keywords:** *in vivo* embryo, MAP5, superstimulation.

The present study evaluated the superovulatory response and *in vivo* embryo production in lactating and non-lactating Holstein donors, treated with a superstimulation protocol using a slow release (MAP5; hyaluronic acid, Bioniche). The study was performed in a commercial dairy farm (Agrindus S/A, Descalvado - SP). A total of 59 donors (29 lactating and 30 non-lactating cows) were initially allocated into two experimental groups in a cross-over design, in a total of 119 superovulations (SOV). At a random day of the estrous cycle (D0), the animals received an intravaginal progesterone device (P4; Primer, Tecnopec; non-lactating cows - 1 device / lactating cows - 2 devices) and 2.0 mg intramuscular (IM) of estradiol benzoate (RIC-BE, Tecnopec). From D4 on, the FOLL group received Folltropin (Tecnopec; n=59; non-lactating cows=300 mg; lactating cows=400 mg) diluted in 20 ml of saline and fractionated in 8 decreasing doses (4 ml, 4 ml, 3 ml, 3 ml, 2 ml, 2 ml, 1 ml and 1 ml), 12 h apart. The MAP/FOLL group (n=59) received the same dose of Folltropin, however, diluted in MAP5 and fractionated in 2 decreasing doses (lactating cows - 6.5 and 3.5 ml; non-lactating cows - 5.0 and 2.5 ml) on days D4 and D6 of protocol. On D6 (AM and PM) 0.150 mg of cloprostenol (Estron, Tecnopec) was injected. On D7 pm, the P4 implants were removed and 12 and 24 h later 62.5 µg IM of lecorelin (Gestran, Tecnopec) was injected in non-lactating and lactating cows, respectively. The non-lactating females were inseminated on D8 PM and D9 AM and the embryo flushing was performed on D15; the lactating females were inseminated on D9 AM and PM and the embryo flushing was performed on D16. Immediately before flushing, the number of corpus luteum (CL) was evaluated and recorded. The sire was the same in both superovulation protocols for each female. Statistical analysis was performed by the GLIMMIX procedures of SAS. There was no interaction between lactating and non-lactating categories for the response variable analyzed (P>0.05), therefore the data were grouped. It was found that animals submitted to the FOLL group had higher superovulation rate [number of females with two or more CL per number of SOV protocols (FOLL: 89.8% and MAP/FOLL: 45.8%; P <0.0001), total of CL at the day of the uterine flush (FOLL: 10.8 ± 1.1 and MAP/FOLL: 3.7 ± 0.7; P<0.0001), total number of recovered ova [FOLL: 7.9 ± 0.9 and MAP/FOLL: 1.8 ± 0.6; P<0.0001], recovery rate [FOLL: 66.8% (425/636) and MAP/FOLL: 48.9% (107/219); P<0.0001] and the number of viable embryos [FOLL: 3.3 ± 0.6 and MAP: 1.8 ± 0.6; P<0.0001] when compared to the animals of MAP/FOLL group. Thus, it was concluded that the injection of two doses of FSH diluted with MAP5 reduces the efficiency of *in vivo* embryos production program of Holstein donors when compared to the use of FSH diluted with saline and injected in 8 decreasing doses.



A137 OPU-IVP and ET

### **Centrifugation force reduction in Percoll gradients for sperm selection increases the fertilizing capacity *in vitro* in bovine**

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**Keywords:** centrifugation, IVF, sperm selection.

High centrifugal forces have been routinely used for separation of spermatozoa by the Percoll gradient method, to obtain a high sperm concentration. These forces, however, can cause anything from minor damage to sperm, such as reduced motility and vigor, until serious consequences to zygote formation, cleavage, and embryo development. This study aimed to determine the influence of centrifugal forces during the selection of sperm by the Percoll method in sperm recovery, morphofunctional characteristics, fertilizing capacity *in vitro*, and embryo development within the first 48h. Five replicates of semen from four bulls *Bos taurus* were used. After thawing they were evaluated using a phase contrast microscope at 400x magnification for motility, vigor, and concentration, and at 1000x magnification for morphology. The membrane integrity was assessed by hypo-osmotic shock technique, with sodium citrate. The samples underwent centrifugation in Percoll discontinuous gradient (30%, 60% and 90%) with the following forces: F1 (9000 x G) or F2 (2200 x G). Oxidative stress was evaluated by measuring the production of ROS (reactive oxygen species), lipid peroxidation, glutathione (GSH), and activity of superoxide dismutase (SOD). ROS levels in semen were determined by a spectrofluorimetric method using 2', 7'-dichlorofluorescein diacetate (DCF-D). A dose of  $2 \times 10^6$  spermatozoa/ml of each bull was used to IVF. Presumptive zygotes (100/treatment) were denuded and incubated with Hoechst 33342 solution (10 mg/ml) and evaluated for the presence of spermatozoa penetrated, formation of the pronuclei or nuclei fused. Some of the presumptive zygotes (10/treatment) were individually cultured for 48 h in SOFaaci + 10% ESS and BSA in petri dishes over an embryonic monitoring system (Primo Vision, Cryo Management Ltd., Hungary). Embryonic development was assessed by the cleavage rate, time of first cleavage, and average number of blastomeres at 48 h. Data were analyzed by chi-square ( $\chi^2$ ) and ANOVA and the means were compared by Tukey test at 5%. There were no significant differences between F1 and F2 in the morpho-functional characteristics, evaluation of oxidative stress, cleavage (87 and 78%, respectively), moment of first cleavage (31.9 and 31.7 h) or average number of blastomeres at 48 h (3.3 and 3.6 cells). However, the F1 produced penetration and normal fertilization rates (203/324, 63%) smaller than F2 (241/354, 68%,  $p < 0.05$ ). These results show that the force of 2200 x G increases the penetration and fertilization rates without reducing the sperm recovery when compared to 9000 x G, that is routinely used in sperm selection by Percoll in cattle.



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### The effect of FSH stimulation prior to the ovum pick-up in *in vitro* embryo production of Gir cows

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**Keywords:** *in vitro* embryo, lactating Gir cow, superstimulation.

The study evaluated the effect of the FSH (Folltropin, Tecnopec) diluted in slow release solution MAP5 (hyaluronic acid, Bioniche<sup>®</sup>) prior to the Ovum Pick-Up (OPU) on *in vitro* embryo production (IVP) program of lactating Gir cows. A total of 12 Gir (*Bos indicus*) cows was used in a *cross-over* design. Females were randomly allocated into three groups: control group (CON; n=12); Folltropin 133 mg (FOLL; n=12); MAP5/Folltropin 133 mg (MAP5/FOLL, n=12). At a random day of the estrous cycle (D0 AM) all cows received an intravaginal progesterone device (P4; Primer, Tecnopec) and 2.0 mg I.M. of estradiol benzoate (RIC-BE, Tecnopec). On D4 and D5 (AM and PM), the FOLL group received four decreasing doses of FSH I.M. (D4 AM and PM = 4ml; D5 AM and PM = 3 ml). The MAP5/FOLL group received a single dose of 3 ml I.M. of MAP5/Folltropin on D4 AM. The P4 devices were removed at D7 AM and cows were submitted to OPU at same day. The same mating was maintained for all IVP procedures during the experiment. The *In vitro* produced embryos (n= 125; CON: n=33, FOLL: n=42 and MAP5F/FOLL: n=50) were transferred to crossbreed recipients previously synchronized for timed embryo transfer (TET). Pregnancy diagnosis was performed 60 days after TET. Variables were analyzed by the GLIMMIX procedure of SAS. The groups FOLL (16.3±1.3) and MAP/FOLL (16.7±1.3) presented higher number of oocytes recovered than CON group (10.9±1.0; P=0.0001). Similarly, higher number of viable oocytes was found in FOLL (11.3±0.8) or MAP5/FOLL (12.9±1.0) than the CON group (7.8±0.5; P<0.0001). However, no differences were found on the blastocyst rate (CON: 35.7±3.2; FOLL: 31.8±3.9; MAP5/FOLL: 28.7±3.3; P=0.44), number of embryo produced per OPU session (CON: 2.8±0.3; FOLL: 3.5±0.4; MAP5/FOLL: 4.2±0.6; P=0.14) and number of pregnancy achieved per OPU session (CON: 1.3±0.6; FOLL: 1.6±0.4; MAP5/FOLL: 1.9±0.2; P = 0.48). In conclusion, FSH treatment prior to the OPU, with or without MAP5, increased the number of oocytes recovered; however, similar number of viable embryos was produced per OPU session in Gir cows.

**Acknowledgments:** Tecnopec.



A139 OPU-IVP and ET

### **Spermatozoa pre-incubation increases the IVF embryo development from poor quality porcine oocytes**

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**Keywords:** IVP, polyspermy, sperm capacitation.

An important cause of low efficiency in porcine *in vitro* embryo production is the high polyspermy rate, which is exacerbated in poor quality oocytes. It was already shown that the reduction in the insemination dose during IVF decreases the rate of polyspermy. It is possible that the sperm pre-capacitation can result in greater polyspermy reduction, mainly in poor quality oocytes. The objective of this study was to evaluate different sperm pre-capacitation periods in porcine IVF of low quality oocytes. Follicles of 3 to 6 mm of diameter were aspirated from ovaries obtained in abattoirs. Low quality oocytes (without compact cumulus cells and heterogeneous cytoplasm; with vesicles and granules) were selected to the study. The IVM was performed in TCM-199 supplemented with 26.19 mM de Sodium Bicarbonate, 25% de Follicular Fluid (FF), 0.1 mg/mL L-Cysteine, 10 ng/mL of Epidermal Growth Factor, 100 UI/mL Penicillin G, 0.1 mg/mL Streptomycin Sulfate, 0.5 mg/mL LH, 0.01 UI/mL FSH and 1 mM dbcAMP. After 22h the oocytes were transferred to an IVM media without LH, FSH and dbcAMP, for additional 18 to 20h. The oocytes were then transferred to dishes with mTBM added with 0.4 mg/mL caffeine and 2 mg/mL BSA. The semen was obtained from fresh ejaculate of an IVF pre-tested boar, and maintained at 15 to 17°C. For fertilization, semen was heated at 30°C for 10 min and sperm selection performed by mini Percoll gradient (45 and 90%). Then the semen was pre-incubated in IVF medium for different periods of times, according the experimental group: 0h (Control), 0.5h, 1h, 1.5h, with 03 replicates. At the end of pre-incubation time, the oocytes were introduced in the medium containing 62,500 spermatozoa/mL and incubated for additional 3h. After this, the zygotes were cultured in PZM-3, being added 10% of FBS in day 4 of culture. IVM, IVF and IVC were performed in an incubator at 38.8°C, at 5% CO<sub>2</sub> atmosphere. The cleavage and blastocyst rates were evaluated at days 2 and 7 of IVC, respectively. The results were analyzed by qui-square test ( $P < 0.05$ ). For cleavage, the pre-incubation groups of 1h (78.8%-190/241) and 1.5h (79.9%-207/259) were similar, and both were higher than 0h (38.1%-45/118) and 0.5h (68.7%-145/211) groups. The higher blastocyst production was observed with 1.5h of pre-incubation (13.5%-35/259). The blastocyst production of the groups 0h (5.9%-7/118), 0.5h (7.6%-16/211) and 1.0h (6.6%-16/241) do not show any differences ( $P > 0.05$ ). The results show that 1.5h of sperm pre-incubation prior to IVF increases the cleavage and blastocyst rates of low quality porcine oocytes. In conclusion, adjusts in IVP system can result in a satisfactory embryo production with low quality porcine oocytes.



A140 OPU-IVP and ET

### ***In vitro* production of bovine embryos after meiosis delay**

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**Keywords:** forskolin, *in vitro* maturation, meiosis.

This study aimed to show if the use of forskolin is able to inhibit maturation in bovine oocytes, producing a higher rate of embryos *in vitro*. Nellore oocytes were matured in TCM-199 and to delay meiosis, the oocytes were maintained for 6 h in medium in presence of 0.1mM forskolin. Then the oocytes were cultured for 18 h in agent-free medium to resume meiosis, completing 24 h of maturation. After resume of meiosis, the oocytes were stained with Hoechst 33342 and classified in: germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), metaphase II (MII), degenerated or unidentified (D/U). Then (day 0), oocytes were fertilized in human tubal fluid (HTF) and the semen was selected by Percoll gradient and the concentration adjusted to  $2 \times 10^6$  sperm/mL. The presumptive zygotes were culture in SOFaa in 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N<sub>2</sub> atmosphere until day 7, when blastocyst rate was evaluated. Apoptosis in blastocysts was accessed by TUNEL (Terminal deoxynucleotil transferase Uracil Nick End Labeling) reaction. Data were analyzed by ANOVA, followed by Tukey test using the general linear model (PROC GLM) of SAS. The level of significance was 5%. There were differences in MI phase between the control group:  $8.3 \pm 6.2^a$  (N=166) and forskolin 0.1mM group  $34.1 \pm 6.7^b$  (N=144);  $P < 0.05$ . On the other hand, there were no differences in other phases between the control group (GV: 0, GVBD:  $0.8 \pm 0.9$ , MII:  $67.6 \pm 9.6$ , D/U:  $7.3 \pm 3.8$ ) and forskolin 0.1mM group (GV: 0, GVBD:  $1.0 \pm 0.9$ , MII:  $50.2 \pm 10.4$ , D/U:  $14.1 \pm 4.1$ )  $P > 0.05$ . No differences were observed in blastocyst production rate between control ( $36.7\% \pm 3.7$ ) and forskolin 0.1mM ( $25.1\% \pm 3.7$ ) ( $P > 0.05$ ). When we analyzed the apoptosis rate, we found differences between the control ( $6.0\% \pm 6.3^a$ ) and forskolin groups 0.1mM ( $33.4\% \pm 6.3^b$ ) ( $P < 0.05$ ). Although forskolin was able to produce embryos with the same rates of the control group, the embryos treated with this drug presented a higher rate of cellular apoptosis. This suggests that further reduction of the concentration of forskolin used in the maturation medium is needed.

**Acknowledgments:** FAPESP 10/50410-2.



A141 OPU-IVP and ET

### **Superovulatory protocol using low dose of equine pituitary extract (EPE): comparison between Crioula and American Quarter horse breed**

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**Keywords:** donor mares, embryo transfer, follicular growth.

Equine superovulatory protocols frequently lead to a low embryo recovery rate per ovulation (around 50%). The reduction of EPE doses can be an efficient option to increase embryo recovery rates, and also to reduce the costs involved in equine ET procedures. Recently the use of biotechnologies was allowed in Crioula pony mares. This fact has generated a great quest from breeders by biotechniques, especially superovulation and embryo collection. However, there is not a suitable protocol and there is little information about using of these biotechnologies in Crioula breed. This study aimed to evaluate the superovulatory response of embryo donor mares of Crioula and American Quarter Horse (AQH) breeds, using an EPE low doses protocol. Six Crioula and eight AQH mares, with excellent sanitary and reproductive conditions, were submitted to a dairy follicular control by ultrasonography. The animals were distributed into two groups. In the control group the monitoring of follicular growth was performed until the time of natural ovulation. In the EPE group, when one or more follicles reached 20mm in diameter, was initiated the IM injection of 7mg of EPE, twice a day, with 12h interval, until the time of ovulation induction. When follicles reached 35mm of diameter, ovulation was induced by injection of 2500IU of hCG. Embryo donors were inseminated every 48h until ovulation and the embryo recovery were performed at the eighth day post ovulation. The mares were monitored during three consecutive estrous cycles, undergoing both treatments. Data were analyzed with GLM procedure of the SAS statistical package. The variables were analyzed by Tukey's test and the least squares means were adjusted for Tukey-Kramer multiple comparisons test. Crioula and AQH breed's mares showed different follicular growth rate. We observed that in Crioula mares the follicular growth was slower, leading to longer duration of superovulation therapy (6.6 days) compared to the AQH mares (4.7 Days -  $P < 0.01$ ). The number of recovered embryos was influenced by the protocol with low doses of EPE in the Crioula breed ( $P < 0.01$ ), but showed no effect on AQH breed mares ( $P > 0.05$ ). The amount of recovered embryos was higher in Crioula mares treated with EPE ( $3.0 \pm 2.0$  embryos) when compared with not treated counterparts (natural ovulation,  $0.83 \pm 0.4$  embryos), and also compared with the AQH mares treated with EPE ( $1.12 \pm 0.8$  embryos) or not treated (Control  $0.5 \pm 0.5$  embryos) ( $P < 0.05$ ). The superovulatory protocol using low doses of equine pituitary extract increased the number of embryos collected for the Crioula breed mares compared with AQH mares, being an interesting alternative to increase the number of embryos recovered per donor mare.



A142 OPU-IVP and ET

### **Effect of the use of successive hormonal stimulation and laparoscopic oocyte recovery on quanti-qualitative oocyte production in goats raised in the tropics**

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**Keywords:** goat, laparoscopy, oocyte.

The laparoscopic oocyte recovery (LOR) can be an excellent tool for the multiplication of genetically superior goats when associated with *in vitro* embryo production (IVEP). However, for a more efficient technique it is important that it can be performed several times in the same donor. This study aimed to verify the effect of the successive use of hormonal treatment followed by LOR on the quantitative and qualitative production of oocytes in goats raised in the tropics. For this purpose, 12 adult crossbred Anglo Nubian goats, cyclical, were submitted to five successive hormonal treatments for ovarian stimulation. Intravaginal sponges impregnated with 60 mg of medroxyprogesterone acetate (Progespon, Syntex, Buenos Aires, Argentina) were inserted at the beginning of the treatment (D0). On D7 75 µg of cloprostenol (Prolise, ARSA, Buenos Aires, Argentina) was given i.m. The stimulation was obtained by the injection of 180 mg pFSH (Folltropin-V, Bioniche, Belleville, Canada), divided into 5 decreasing doses with an interval of 12 h from D7 to D9 of the progestagen treatment. Thirty-six h before LOR, goats were fasted and LOR was performed using volatile anesthesia, starting with i.v. injection of 20 mg / kg thiopental (Tiopentax 2.5% Cristália, Sao Paulo, Brazil) and maintained with isoflurano (Isoforine, Cristália, São Paulo, Brazil). Under laparoscopic control, the follicles were visualized and classified as small (<3 mm), medium (3-4 mm) and large (> 4 mm). The cumulus-oocyte complexes (COCs) were aspirated with the help of a vacuum system (WTA, Cloves, Brazil) and in classified in Grades I to IV. The results were expressed as mean ± SEM and analyzed by ANOVA (repeated measures) followed by Tukey test (5%). A total of 1149 follicles (19.1 ± 2.6 follicles / donor) was punctured and 822 oocytes were collected (13.7 ± 1.6 oocytes / donor) which resulted in a total harvest rate of 71.5%. There were no statistical differences in the number of punctured follicles and oocytes collected during the five sessions LOR, respectively (21.3 ± 2.1 and 12.9 ± 1.5 vs 21.8 ± 3.4 and 15.6 ± 1.7 vs 20.0 ± 1.7 and 15.2 ± 1.5 vs 16.5 ± 2.3 and 12.9 ± 2.0 vs 16.3 ± 1.4 and 11.9 ± 1.1; P > 0.05). However, the number of large follicles observed in session 1 was different when compared to sessions 2 and 4 (5.8 ± 0.7 vs 2.8 ± 0.6 and 2.0 ± 0.4, P < 0.05). Regarding to harvest rate, there was no statistical difference between the sessions, averaging 61.4, 72.6, 75.5, 71.7 and 73.2% (P > 0.05) for sessions one to five, respectively. Only 17% of oocytes collected were classified as degenerated (Grade IV) and 83% viable (GI-III). In conclusion, five successive hormone treatments followed by LOR did not affect the quantitative and qualitative production of oocytes in goats rose in the tropics, and may be a useful tool for genetic improvement in goats.



A143 OPU-IVP and ET

### **Use of forskolin as lipolytic drug production and cryopreservation of bovine embryos produced *in vitro* (PIVE)**

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**Keywords:** bovine embryos, cryopreservation, forskolin.

Bovine embryos produced *in vitro* have less tolerance to cryopreservation compared to embryos produced *in vivo*. One of the factors related to this low tolerance is the highest concentration of intracellular lipid in *in vitro* embryos. The forskolin is a substance derived from an Indian plant (*Coleus forskohlii*) has been described in the literature as a metabolic regulator in inhibiting the formation of intracellular lipid droplets (Ye, J., et al. 2010, Regul. Pept., 161, 58-66). The aim of this study was to evaluate the effect of forskolin on the rate of blastocyst and their respective cryopreservation. Oocytes were recovered from abattoir ovaries, matured for 22 h (TCM 199 with 10% FCS, 5.0 mg / mL LH 0.5 mg / mL of FSH, 0.2 mM pyruvate and 50 g / mL gentamicin) for 18 h fertilized (TALP with heparin and PHE -  $2 \times 10^6$  spermatozoa / mL) and cultured for 7 days in SOFaa (10% FCS) supplemented with 0 (control), 10, 25, and 50 mM forskolin 38.5°C and 5% CO<sub>2</sub>. On the seventh day of culture was determined blastocyst rate compared to the total number of oocytes and embryos in the blastocyst stage expanded (grade I and II) were cryopreserved by slow freezing technique, using ethylene glycol as a cryoprotectant. After freezing and thawing, embryos were co-cultured in 100mL of droplets with SOFaa cell monolayer incubated at 38.5°C, 5% CO<sub>2</sub> and determined the percentage of re-expansion (24 h), and hatch (48 h). Data were analyzed by ANOVA followed by Tukey test ( $P < 0.05$ )  $\pm$  standard deviation. The embryonic development of 240 oocytes were evaluated in four replications. The percentage of embryonic development on the seventh day of culture in medium supplemented with 0, 10, 25 and 50 mM forskolin was ( $40.08 \pm 6.0$ ,  $48.26 \pm 21.26$ ,  $44.42 \pm 6.0$  and  $47.42 \pm 8.34\%$  respectively), showing no statistical difference ( $P > 0.05$ ). The evaluation of the rate of re-expansion of the embryos treated with forskolin did not differ ( $P > 0.05$ ) between the control group ( $86.0 \pm 8.28\%$ ), 10 ( $17.13 \pm 81.0\%$ ), 25 ( $83.0 \pm 5.3\%$ ) and 50 mM ( $17.2 \pm 76.0\%$ ). The percentage of embryos hatched after thawing was  $56.0 \pm 14.70$ ,  $74.0 \pm 25.21$ ,  $55.0 \pm 17.57$  and  $25.35 \pm 28.0\%$  for the groups treated with 0, 10, 25 and 50 mM forskolin, respectively, with no significant difference ( $P > 0.05$ ). In conclusion, bovine embryos cultured in the presence of different concentrations of forskolin did not increase the rate of embryo cryopreservation and tolerance in relation to the control group.



A144 OPU-IVP and ET

### **Pregnancy rates following FTET of *Bos taurus* x *Bos indicus* with high, intermediate and low numbers of antral follicles**

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**Keywords:** antral follicle count, embryo recipient, pregnancy.

The objective of this study was to evaluate the influence of the antral follicles population on pregnancy rate of embryo recipient heifers. *Bos taurus* x *Bos indicus* heifers (*Bos indicus*; n=281) with BCS of  $3.0 \pm 0.5$  were submitted to a protocol for synchronization of ovulation. The animals received an intravaginal device (CIDR, Zoetis, Brazil) and 2mg of estradiol benzoate (EB); (Estrogin, Farmavet, Brazil). Eight days later, the animals received 0,5 mg of PGF2 (Ciosin, Intervet Schering-Plough, Brazil), 300UI of eCG (Novormon, Syntex SA, Argentina) and 0.5 mg of estradiol cypionate (EC);(ECP, Pfizer, Brazil), IM. Antral follicles  $\geq 3$  mm were counted (D17) using an intravaginal micro-convex array transducer and the heifers received embryos produced in vitro 17 days after the beginning of the hormone treatment. Heifers were assigned into groups with high antral follicular count (AFC; G-High  $\geq 30$  follicles, n=38) intermediate AFC (G-Intermediate,  $< 11$  follicles, n = 136) or low AFC (G-Low,  $\leq 3$  follicles, n = 75). The number of follicles was evaluated by Kruskal-Wallis and pregnancy rates were compared by Chi-square test ( $P \leq 0.05$ ). The average number of antral follicles (mean  $\pm$  SD) was  $12.94 \pm 9.21$  and the mean pregnancy rate 33.08% (93/281). The mean population of antral follicles was  $25.82 \pm 7.40$  (G-High),  $11.34 \pm 2.96$  (G-Intermediate) and  $3.85 \pm 1.35$  follicles (G-Low,  $P < 0.05$ ). There was no difference in pregnancy rates between groups (G-High, 30.00%; G-average, 33.82%, G-Low, 34.60%,  $P > 0.05$ ). It was concluded that the population of antral follicles on D17 of the TETF protocol does not affect the pregnancy rate of embryo recipient heifers.



A145 OPU-IVP and ET

### **Evaluation of aqueous extract of propolis as replication inhibitor of murine zygotes *in vitro* experimentally infected by bovine herpesvirus type 1 Colorado strain (BoHPV-1)**

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**Keywords:** BoHPV-1, propolis, replication inhibitor.

Despite greater control over the transmission of pathogens by the use of biotechnologies in animal breeding, there is, paradoxically, the potential for disease transmission by bovine embryo transfer which remains a justifiable concern for regulatory agencies. The objective of this work was to evaluate whether the detrimental effects of the experimental infection of murine zygotes with bovine herpesvirus type 1 (BoHPV-1; Colorado strain,  $10^8$  TCID<sub>50</sub>/mL) during 24 h could be reduced by the viral inhibitor aqueous extract (AE) of Propolis (Pp). Between six and eight weeks old female mice (Swiss lineage) were superovulated with 0.2 mL (5 UI) of eCG-hCG and mated with males from the same lineage. After 18 h, the zygotes were collected and washed with 0.5% pronase solution. The zygotes were allocated into four groups, which were exposed to: 10 µL of PBS (G1; control), 10 µL of BoHV-1 virus (G2;  $10^8$ /mL TCID<sub>50</sub>/mL), 10 µL of Propolis Aqueous Extract (PpAE) in 0.001% in PBS (G3), 10 µL of PpAE in 0.001% in PBS and 10 µL of BoHV-1 virus (G4). All groups were kept for 24 h at 37.5°C, 5% CO<sub>2</sub> in air. In this work were evaluated the embryo morphological differences, cleavage rate, and viral replication by titration (Reed and Muench test) after 72 h co-culture with Madin-Darby bovine kidney (MDBK) cells. Differences among groups in cleavage rate were compared by Fisher test and in titration by Mann-Whitney test. The non toxic Propolis Aqueous Extract concentration was 0.001%. Only the group G2 showed morphological differences, like granulations and degenerative appearance. The cleavage rates were as follows: G1, 77% (154/201); G2, 53% (138/262); G3, 82% (224/273) and 74% (185/250). There were no significance differences ( $p < 0.005$ ) in cleavage rate among the groups G1xG4 and G1xG3. There was significance difference ( $p < 0.05$ ;  $P = 0.0286$ ) in viral replication rate between groups G2 ( $6.04 \times 10^7$  TCID<sub>50</sub>/mL) and G4 ( $1.63 \times 10^3$  TCID<sub>50</sub>/mL). These results support the conclusion that Propolis Aqueous Extract reduces the viral rate of replication without interfering in cellular development, and may be an alternative to sanitary control protocols.

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A146 OPU-IVP and ET

### Seasonal variation of OPU-IVP results in Zebu donors (*Bos taurus indicus*)

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**Keywords:** climate, oocytes, Zebu.

Zebu breed donors produce more oocytes and are less sensitive to seasonal variations in the results of *in vitro* production of embryos in tropical and subtropical regions (Viana, 2005. *Acta Sci. Vet.*,33, 213-219; Camargo *et al*, 2007, *Theriogenology*, 68, 626-632; Nunes. 2010, *Reprod. Fertil. Dev.* 22, 567-573.). This study aimed to compare the distribution of the results of IVEP over the months of the year in *Bos taurus indicus* donors, done in the south and southwest of the Minas Gerais state (Brazil) by the same veterinarian. The region has mesothermic climate of CW classification, according Koepen. Results from 1349 follicular aspirations (OPU) from Gir (594), Nelore (345) Brahman (283) e Guzará (127) donors were used, done for a period of 12 months. In this period the total rainfall was 2.970mm, the maximum temperature was 38.7°C and the minimum was 4.6°C. The management of animals was in the pasture (*Brachiaria decumbes e Brachiaria brizanta*) with commercial mineral supplementation *ad libitum*. For OPUs was used an ultrasound device with intravaginal microconvex transducer of 7,5 MHz (Mindray DP 2200). All follicles larger than 3 mm were identified and punctured. The Cumulus oocytes complexes (COCs) recovered were counted and classified based on their morphological aspect. The viable oocytes were matured (TCM 199) for 22-24 hours after the start of OPU at a temperature of 38.5°C, 5% CO<sub>2</sub> and saturated humidity. Sorted sexed semen of different bulls, evaluated for motility and vigor, was used for *in vitro* fertilization (IVF). After IVF the presumptive zygotes were transferred to *in vitro* culture (SOF), where they remained for seven days at a temperature of 38.5°C and controlled atmosphere (5% CO<sub>2</sub>). The average production of oocytes and embryos conversion was compared between breeds and months of the year by Dunn's test, considering a 5% significance. There was no breed effect for any variable. Donors produced an average of 20.4±16.5 total oocytes, 13.2±12.5 viable oocytes, and 4.0±3.2 embryos per OPU. No differences were observed in the production of viable and totals oocytes in the different months of the year. In April and May, the average production of embryos was lower (P<0.05), observed by a lower conversion of oocytes into embryos in those periods. The drop in efficiency of zebu donors during this period may be related to two situations simultaneously: possible cold stress (Hansen, 2004. *Anim. Reprod. Sci.* 83, 349-360) due to drop temperature during these months and indirect effect by reducing the quality of pastures (Turner 1980, *J.Anim.Sci.* 50, 1201 – 1205). It was concluded that zebu donors suffer seasonal variation in the intrinsic quality of the oocyte and embryo production by IVP in the region studied.

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A147 OPU-IVP and ET

### Effect of HSP90 inhibitor on developmental competence of bovine oocytes

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**Keywords:** 17AAG, heat shock, Maturation *in vitro*.

The heat shock protein 90kda (HSP90) is a cytoprotective chaperone that influences the maturation of *Xenopus* oocytes (Nebreda and Ferby, 2000. *Curr Opin Cell Biol* 12:666-675). Its effect can be repressed by inhibitors as 17-(allylamino)-17-demethoxygeldanamycin (17AAG, Sigma, St. Louis, USA). This study aimed to evaluate the effect of 17AAG concentration and exposure time during *in vitro* maturation in order to identify a possible HSP90 requirement for oocyte developmental competence. Immature oocytes aspirated from ovaries obtained from slaughterhouse were selected and randomly allocated in a factorial experiment design with three 17AAG concentrations (0, 1 and 2 $\mu$ M) and two-exposure time (12 and 24h) during *in vitro* maturation. The maturation was performed in Nunc plate containing 400 $\mu$ L of TCM199 medium (Invitrogen, Carlsberg, USA) supplemented with porcine FSH (pFSH - Pluset, Lab. Callier, Espanha) and 10% estrus cow serum, and incubated at 38.5°C under 5% CO<sub>2</sub> and 95% humidity for 24h. Oocytes were *in vitro* fertilized (IVF) for 20h and incubated under the same maturation conditions. Semen was processed by Percoll gradient and a fertilizing dose of 2x10<sup>6</sup>spermatozoa/mL was used. After IVF, the presumptive zygotes were denuded in a solution of PBS plus 1% hyaluronidase and then cultured in wells with 500  $\mu$ L of modified CR2aa medium supplemented with 2.5% FCS (Nutricell, Campinas, Brasil) in an incubator at 38.5°C under 5% CO<sub>2</sub>, 5% CO<sub>2</sub> and saturated humidity for 8 days. Cleavage was evaluated on day three (D3) and blastocysts were evaluated on day seven (D7) and on day (D8) post-fertilization. Data from nine replicates (n=1836 oocytes) were analyzed by Generalized Linear Model procedure of SAS software (version 9.1) considering effect of exposure time, 17AAG concentration and interaction, and means were compared by Student Newman Keuls test. Values are shown as mean  $\pm$  SEM. Regarding to exposure time, there was no difference for cleavage and blastocysts rates in D7 and D8 between 12h and 24 hours. Blastocyst rates of 2 $\mu$ M 17AAG group were decreased on D7 (18.6 $\pm$ 2.2%; P<0.02) and on D8 (20.4 $\pm$ 2.2%; P<0.01) when compared to 0 $\mu$ M (29.2 $\pm$ 2.5% and 34.0 $\pm$ 3.3% for D7 and D8, respectively), whereas 1 $\mu$ M produced intermediary blastocyst rates (25.6 $\pm$ 2.7% and 27.9 $\pm$ 3.1% for D7 and D8, respectively). There was no interaction (P>0.05) between concentration and exposure time. In conclusion, inhibition of HSP90 by 17AAG decreases oocyte developmental competence and suggests that this protein is also required for maturation of bovine oocytes.

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A148 OPU-IVP and ET

### Use of reverse sorted and conventional semen on “*in vitro*” embryo production

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**Keywords:** cleavage, embryo, sexing.

Sexed semen production using previous frozen straws (reverse sorted semen), allows produce animals of desired gender, even after male death or sterility. It is known that semen sexing is harmful to spermatozoa function (Seidel, 2002, *Reproduction*, 124, 733–743); however, the possibility to have more embryos from desired sex made the reverse sorted semen available to commercial laboratories of embryo IVP. However, literature data comparing embryo IVP using conventional or reverse sorted semen are rare. The aim of our work was to compare the embryo *in vitro* development rates using conventional or reverse sorted semen from same animals (6 bulls). To achieve our goal we used data from an embryo IVP commercial laboratory during 2011 and 2012. Results were analyzed by ANOVA with 5% significance level (SAEG). A total of 178 ovum pick-ups were performed in conventional semen group and 85 in sex sorted semen group, whereas 2692 e 1536 viable oocytes were obtained, respectively. No differences were observed between cleavage rate:  $67\% \pm 2$  and  $70\% \pm 3$ , or embryonic production rate seven days after fertilization:  $36\% \pm 2$  and  $34\% \pm 3$ , in conventional and sexed sort semen, respectively. Once that a larger number of oocytes were used and the same bulls were distributed in two treatments, it is possible to conclude that injuries caused to bulls gametes during reverse sorting process was not able to decrease *in vitro* embryo development rates.

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**Financial support:** Fapemig.



A149 OPU-IVP and ET

### **Influence of the inhibition of phosphodiesterases-3 and -8 on meiosis resumption of bovine oocytes**

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**Keywords:** cAMP, maturation, phosphodiesterases.

Oocyte in vivo maturation is a highly orchestrated process in which meiosis is resumed by the gonadotropin surge that precedes ovulation and induces the decrease in cAMP levels in the oocyte (Gilchrist, R. B et al., 2009 *Reprod. Fertil. Dev.* v.22, p. 293-293). cAMP is synthesized by adenylyl cyclase (*ADCY*) and degraded by phosphodiesterases (PDE), of which some are cAMP-specific and other to cGMP (Richard *et al.*, 2007, *J Anim Sci*, 85, 4-6). The activity of cAMP-specific PDE3 is prevalent in oocytes, while PDE8 is prevalent in cumulus cells (Sasseville *et al.*, 2009, *Biol Reprod*, 81, 415-425). The aim of the present study was to determine the contribution of different isoforms of phosphodiesterases in resumption of meiosis when high levels of cAMP are maintained by the addition of different cAMP-specific PDE inhibitors to the maturation medium. Cumulus-oocyte complexes (COCs) were matured in vitro in the presence, absence or combination of inhibitors of cAMP-specific PDEs. COCs were aspirated from abattoir ovaries, selected (grade 1 and 2), transferred in groups of 10 to 100  $\mu$ L droplets of TCM 199 + 0.1% PVA, under mineral oil, containing the treatments: 1) control (no inhibitors), 2) PDE3 inhibitor (cilostamide, 20 $\mu$ M) 3) PDE8 inhibitor (dipyridamole, 50 mM) and 4) combination of cilostamide and dipyridamole. Cultures were carried out for 9 h at 38.5°C and 5% CO<sub>2</sub> in air. For evaluation of nuclear maturation rates, the oocytes were denuded, fixed between slide and cover slip for 24 h (methanol: acetic acid, 3:1), stained with 1% acetic orcein and observed under phase contrast microscope. Oocytes in germinal vesicle (GV) were considered immature and in metaphase I (MI) as having already resumed maturation (GV breakdown). Six replicates were performed. Data analyzed for the effects of the treatments were tested by ANOVA and means compared by Tukey test (SAS, 2002), considering a level of significance of 5%. Cilostamide alone or in combination with dipyridamole reduced the proportion of oocytes reaching MI (20.9 and 28.7%, respectively) compared to control (77.7%,  $P < 0.05$ ) and dipyridamole (74.8%,  $P < 0.05$ ). Cilostamide combined with dipyridamole was similar to cilostamide alone ( $P > 0.05$ ). In conclusion, the inhibition of PDE3 (most active isoform in oocytes) with cilostamide was sufficient to reduce the proportion of oocytes resuming meiosis. Inhibition of PDE8 (most active isoform in cumulus cells) with dipyridamole did not inhibit meiosis resumption and its association to cilostamide did not contribute further for the meiosis inhibition induced by cilostamide alone. Therefore, it seems that there is a major relative contribution of cAMP levels produced by oocytes to block meiosis than of cumulus cells. However, more studies are necessary to confirm this possibility.

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A150 OPU-IVP and ET

### Effect of recombinant human FSH during *in vitro* maturation on apoptosis of bovine blastocysts

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**Keywords:** apoptotic index, maturation, recombinant hormone.

Previous study reported that recombinant human FSH (rhFSH) during *in vitro* maturation (IVM) allows blastocyst production at the same rate of FSH from porcine pituitary (Souza et al., 2012. Anim Reprod, 9:550). The present study aimed to evaluate the total cell number and cells in apoptosis in blastocysts generated from oocytes matured *in vitro* with different rhFSH concentrations. rhFSH was gently donated by Galactous Biotech Ltda (Concepción, Chile). Immature COCs obtained from slaughtered animals were randomly allocated in six treatments of *in vitro* maturation as follow: T1 - control with porcine FSH (pFSH - Pluset, Callier, Spain), T2 – without FSH; T3 – 0.0105 UI rhFSH; T4 – 0.021 UI rhFSH; T5 - 0.042 UI rhFSH and T6 – 0.084 UI rhFSH. IVM was performed in TCM199 medium (Invitrogen, Carlsberg, USA) supplemented with 10% estrus cow serum for 24h at 38.5 °C under 5% de CO<sub>2</sub> and 95% humidity. After IVM, oocytes were *in vitro* fertilized and cultured in modified CR2aa medium supplemented with 10% fetal calf serum (Nutricell, Campinas, Brasil) at 38.5 °C under 5% de CO<sub>2</sub> and 95% humidity. Two hundred two blastocysts, from the different treatments, were fixed in 4% paraformaldehyde at the eighth day post-fertilization and evaluated by TUNEL technique for quantification of cells number and apoptotic index. Evaluation of total cell number and apoptotic index of inner cell mass (ICM) and trophoblast (TE) were also performed in some blastocysts (n=45). Localization of ICM and TE nuclei was based on their position in the captured images using ImageJ software. Data was submitted to analysis of variance and means compared by Student Newman Keul's test. Values are shown as mean ± SEM. There was no difference (P>0.05) on total cell number, total apoptotic cell number and apoptotic index in blastocysts from different treatments. When only TE was evaluated, both the total number of cells and the apoptotic index also were not different between treatments. However, in ICM, no difference in total number of cells was observed, but the blastocysts from T1 and T2 showed lower (P<0.05) apoptotic index (28.8±5.8 and 29.4±3.9%, respectively) than blastocysts from T3 and T4 (57.8±8.3 and 58.6±7.3%, respectively) but similar to T5 and T6 (44.8±4.4 and 46.3±3.8%, respectively). In all treatments the index of apoptotic cells in ICM (44.6±2.6) was higher than in TE (10.6±0.9). Overall, rhFSH during IVM does not influence the cells number or the index apoptotic in bovine blastocysts, nevertheless, can increase the apoptotic rate of ICM.

**Financial support:** FAPEMIG and CNPq.



A151 OPU-IVP and ET

### **Effect of ethylene glycol and glycerol in slow freezing of bovine embryos produced *in vitro***

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**Keywords:** ethylene glycol, glycerol, slow freezing.

For embryo freezing the use of intracellular cryoprotectants is recommended to protect the embryos from temperature decrease and ice crystal formation during the process. The intracellular water is replaced for the cryoprotectant while the temperature decreases. (Rodrigues, 2011; Nieman and Sommerfeld, 1999). The objective of the present work was to evaluate the viability of embryos produced *in vitro* after slow freezing with ethylene glycol or glycerol. Embryos were produced in accordance with the standardized protocol of Vitrogen. Expanded blastocysts of seven days in culture were selected and randomly divided into two groups (ethylene glycol and glycerol): Group 1 (n = 56) - exposed to ethylene glycol 1.5M with 0.4% BSA and 0.1M sucrose; Group 2 (n = 137) - exposed to glycerol in three steps: 1) 0.4% BSA (BSA) in Embriolife medium; 2) 5% Glycerol, with 0.1M sucrose in Embriolife medium, and 3) 10% Glycerol, with 0.1M sucrose in Embriolife medium, with exposure to glycerol of 5 minutes. Slow freezing was performed with the TKR (PK1000 model machine), in which each straw (0.25 mL) had seven embryos. All groups were submitted to cooling rate of 1 to 3°C/min until the temperature of -60°C, when crystallization was induced. Cooling continued to 0.3°C/min up to 35°C negative, when the straws were immersed in liquid nitrogen (N<sub>2</sub>), and stored in liquid nitrogen containers. Thawing of Group 1 and Group 2 were done in two steps: five seconds in the air and 15 seconds in water bath between 19-20° c. After this period, embryos received two five-minute baths, in two separate drops containing culture medium for removal of cryoprotectant, and returned to incubator at 38.5°C in a humidified atmosphere of 5% CO<sub>2</sub> in air for evaluation of reexpansion and hatching rates at 24, 48 and 72 hours post-thawing. Statistical analysis was performed using the Student t-test with a significance level of 5%. Reexpansion rates in the first 24 hours post-thawing were not different; however, at 48 and 72 hours after thawing, Group 1 (ethylene glycol) was better when compared with Group 2 (glycerol). In Group 1, the best result concerning reexpansion was obtained with 72 hours after thawing (35.7%). In Group 2, the best rate was obtained with 24 hours after thawing (19%). The bovine embryos produced *in vitro* and frozen slowly in medium containing ethylene glycol had higher reexpansion rate when compared to embryos frozen in the medium containing glycerol.



A152 OPU-IVP and ET

### **Effect of ovarian stimulation using FSH on oocyte recovery rate and *in vitro* embryos production in Sindhi breed - Preliminary results**

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**Keywords:** in vitro fertilization, OPU, ovarian superstimulation.

The oocyte competence acquisition can be stimulated by FSH administration before follicular aspiration (FA) (Nivet et al., 2012, *Reproduction* 143, 165-171; Chaubal et al., 2007, *Theriogenology* 67, 719 - 728, Blondin et al. 2002, *Biol Reprod* 66, 38-43). The time between ovarian stimulation by the application of exogenous gonadotropins and FA (coasting), as well as the stage of follicular wave during ovarian overstimulation are crucial to the success of the technique (Chaubal et al., 2007, *Theriogenology*, 67, 719-728). The objective of this study was to compare the effect of ovarian stimulation using FSH before follicular aspiration in Sindhi cows on oocyte recovery rate and *in vitro* embryo production. Thirteen cyclic females were randomly distributed in three treatments. In all animals was performed ablation of follicles larger than five millimeters in diameter at the beginning of the treatment (D0). The animals were kept with intravaginal implant containing 1.0 g of natural progesterone (Cronipres®, Biogenesis-Bagó SA, and Buenos Aires, Argentina) during all over the experiment, being removed only at the time of FA. In all animals a new progesterone implant was introduced in vagina just after FA. The treatment groups were: T1- Ovarian stimulation with FSH 80 mg (Folltropin-V®, Bioniche, Belleville, Ontario, Canada) divided in three intramuscular decreasing doses and with 24 h interval between applications (D1, D2, D3), being the last dose 54h before FA (D5,5); T2-Ovarian stimulation with FSH 80 mg, with a single dose (half dose administered subcutaneously and other half intramuscularly) 102h (D1) before FA and simultaneous with the first application of the T1 group; T3- Control group without ovarian stimulation. The hormonal administration began in the day after follicular ablation (D1). Three FA sessions for each treatment were performed within 1-week intervals between sessions. Data were analyzed by ANOVA and Tukey test. The number of follicles aspirated was 10.77±5.91 in T1, 10.22±6.41 in T2 and 9.87±1.88 for the control group. The mean of oocytes retrieved was 9.00±7.21 in T1, 8.11±2.39 in T2 and 6.00±2.39 in control group. The oocytes recovery rates were 83.50% (81/97) 79.34% (73/92) and 60.75% (48/79) respectively for the groups T1, T2 and control. Cleavage rates were 59.25%, 67.12%, 72.91% and blastocyst rates were 31.2%, 35.61% and 27.08%, respectively for T1, T2 and control group. No differences were found between groups for the characteristics evaluated. The FSH doses were not sufficient to increase the oocyte recovery rate and embryos production, being to necessary new studies for validation the stimulation protocols in this breed.



A153 OPU-IVP and ET

### Effects of fatty acid supplementation in Holstein cows during the pre and postpartum period on oocyte quantity and *in vitro* production of embryos

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**Keywords:** *bos taurus taurus*, OPU-IVPE, polyunsaturated fatty acids.

The supplementation of dairy cattle with sources of polyunsaturated fatty acids (PUFA) can be used to increase the energy level of the diet (Van Knegsel *et al.*, 2005, *Reproduction Nutrition Development*, 45, 665-688). The PUFA provides positive effects on reproductive functions of important tissues, including the hypothalamus, pituitary, ovaries and uterus (Funston 2004, *Journal of Animal Science*, 82, 154 - 161). The aims of this study were to evaluate the reproductive condition of postpartum, number of follicles, aspirated oocytes, amount of viable oocytes and the *in vitro* production of embryos (IVPE) of the Holstein multiparous donors supplemented with rich diet in protected PUFA (especially linoleic acid - n-6) and non-protected (especially linolenic acid - n-3) during pre and postpartum. The donors were allocated into three groups, a control group (C) with 6 donors (management of the farm, with no fat source supplementation), a Megalac-E group (M) with 5 donors (supplemented with 100g/donor/day in pre-partum and 300g/donor/day in postpartum) and a linseed group (L) with 5 donors (supplemented with 1kg linseed pie/donor/day pre-partum and 1.5 kg/donor/day in postpartum). The Megalac-E is a protected fat source and linseed pie is fat source not protected. The diets had been given for antepartum during thirty days and postpartum sixty days. The animals were submitted to OPU on days 30, 45 and 60 postpartum. The recovered oocytes were selected and the viable ones were submitted to procedures of the IVPE. The data from both experiments were analyzed by the method of least squares using variance analysis of proc GLM. The differences between means were compared by Tukey test with 5% significance. There was no detectable effect of treatment and aspirations of postpartum days on variables: amount of viable oocytes and viable oocytes rate (C=3.38±1.22 and 59%; M=3.20±1.34 and 70%; L=8.86±1.34 and 72%; P>0.05); IVPE and embryos production rate (C=1.00±0.24 and 29%; M=0.20±0.27 and 6%; L=1.33±0.27 and 15%; P>0.05). However, was observed in the group supplemented with linseed pie more follicles and total oocytes than group Megalac-E (C=11.27±1.49 and 5.72±1.24; M=8.46±1.63 and 4.53±1.36; L=18.33±1.63 and 12.26±1.36; P<0.05). The aspirations performed in postpartum donors supplemented with PUFA didn't increase the number of viable oocytes and IVPE. Thus, more studies are needed with higher numbers of donors and different fat sources to test the real influence of PUFA in dairy females.



A154 OPU-IVP and ET

### Effect of norgestomet on *in vitro* embryo production in *Bos indicus* and *Bos taurus* cows

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**Keywords:** bovine, *in vitro* embryo production, Norgestomet.

The aim of this study was to determine the effect of previously used norgestomet auricular implant or of two new norgestomet implant on *in vitro* embryo production from Gir (*Bos indicus*) and Holstein (*Bos taurus*) breeds. A total of twelve pluriparous, non-lactating cows were selected in this study, 6 Gir and 6 Holstein. The animals were subjected to six follicular aspiration consecutive with fourteen days of interval between OPU. The study was performed in cross-over design, in which cows were subjected alternately to one of the hormonal treatment in each OPU session: control – animals that did not receive auricular implant, low norgestomet – animals receiving used auricular implant of norgestomet (Crestar<sup>®</sup>, Intervet, Brazil), high norgestomet – cow treated with two new norgestomet implant (6 mg of norgestomet combined). For synchronize the emergence of a new follicular wave, all cows received 3 mg of estradiol benzoate (RIC-BE<sup>®</sup>, Tecnopec, Brazil) plus 150 µg D-cloprostenol (Prolise<sup>®</sup>, Tecnopec, Brazil) to eliminated the presence of corpus luteum and endogenous progesterone influence in the treatment. The follicular aspiration was performed 7 days after the beginning of hormonal treatment. The ear devices were removed 24 hours after OPU. All follicles  $\geq 3$  mm were aspirated and the oocytes recovered were morphologically evaluated, selected and those considered as viable were matured for 22-24 hours in TCM 199 medium (Tecgene, Brazil). Then, CCOs were fertilized with semen from bull of known fertility, processed by Percoll discontinuous gradient and gametes were co-incubated during 18-20 hours. After this period, presumptive zygotes were cultured for 7 days in SOFaa medium supplemented with 5% of FCS. Data were analyzed by ANOVA, considering the effects of treatment and genetic group and when a significant effect was obtained ( $P < 0.05$ ), means were compared by Tukey test. A difference was detected in the number of embryos on day 7 for the Gir cows of the low norgestomet treatment than that from control and high norgestomet ( $5.1 \pm 1.2$  vs  $3.0 \pm 0.7$  and  $2.7 \pm 0.6$ , respectively). For performance on *in vitro* embryo production an advantage was observed in the Gir as compared to Holstein cows on number of follicles visualized ( $21.1 \pm 0.7$  vs  $15.7 \pm 0.6$ ), number of recovered oocytes ( $12.7 \pm 1.1$  vs  $8.2 \pm 0.7$ ), number of oocytes grade II and III ( $3.4 \pm 0.4$  vs  $1.8 \pm 0.3$  and  $3.1 \pm 0.4$  vs  $1.6 \pm 0.3$ ), number of viable oocytes ( $9.0 \pm 1.0$  vs  $5.5 \pm 0.7$ ), cleavage rates ( $81.8 \pm 3.5$  vs  $68.7 \pm 4.5$ ) and number of embryos on day 7 ( $3.6 \pm 0.5$  vs  $2.1 \pm 0.4$ ). In conclusion, the present study demonstrated that using an auricular implant of norgestomet previously used in Gir cows, there was an increase in the number of embryos on day 7. Also, Gir breed showed better performance in OPU-IVP program than Holstein cows.

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A155 OPU-IVP and ET

### Seroprevalence of bovine Herpesvirus-1 in embryo recipient cows in the state of Acre

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**Keywords:** embryos, IBR, serological tests.

Infections that directly or indirectly affect the reproductive tract of females and males and compromise the development of embryos and fetuses, promote a great impact on the reproductive efficiency of cattle. Seroprevalence studies conducted in several states in Brazil have shown high percentages of seroconversion for herpesvirus type-1 (BoHV-1) in cows (Fino et al., 2012, Rev. Bras. Reprod. Anim., v36, 122-127). However, until now, there are no studies confirming the presence of BoHV-1 in Acre. Were used 235 crossbred cows (*Bos taurus* x *Bos indicus*) as embryo recipients. All animals received the same protocol for synchronization of ovulation in a program of Embryo Transfer in Fixed Time (FTET). On the 16th day after the beginning of the protocol, an in vitro produced embryo was transferred to each recipient and at the same time a sample of blood was collected by venous puncture of the coccygeal vein in vacuum tubes without anticoagulant. The samples were subjected to centrifugation for obtaining serum. The serological diagnosis of BoHV-1, a virus neutralization test was performed, and animals with title 2 or lesser were considered nonreactive. The diagnoses of pregnancy were performed on the 25th and recipients pregnant were revalued at 55th days after FTET, both by ultrasonography (Aloka SSD 550, Aloka, Japan). The serological diagnosis was performed by the R & D Center of Animal Biological Institute of São Paulo. From the total of samples 43.82% (103/235) were diagnosed as sero-reactive to BoHV-1. The utilization rate of recipients undergoing FTET was 67.23% (158/235) which resulted in 34.17% (54/158) of pregnancy rate, and 37.04% (20/54) of the pregnant recipients had titers against the virus. The revaluation of pregnant recipients on the 55th day, shows that 20.75% (11/54) had aborted and 72.73% (8/11) of these losses occurred in serum-reactive animals, for which the titles of virus neutralization were 1024 (25%), 512 (25%), 256 (37.5%) and 128 (12.5%). The abortion rate, was evaluated by the qui-square test being observed relation between recipients serum positivity and the abortion rate ( $p < 0.05$ ). The high viral concentrations in these recipients show the IBR virus circulation, being responsible for the increased rate of abortion.



A156 OPU-IVP and ET

### **Slaughterhouse and laparoscopic ovum pick up (LOPU) derived goat oocytes have different IVM kinetics and requirements for embryo development**

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**Keywords:** blastocyst, IVF; oocyte maturation.

Oocytes used for goat IVP can be collected either from slaughterhouse (SLAUG) ovaries or by LOPU from live animals. Most studies aiming at setting up and improving goat IVP conditions were performed using SLAUG ovaries and then these results were inferred to LOPU oocytes, whereas oocytes from both sources may have different requirements. A total of 2581 goat oocytes were used. In Experiment 1, the aim was to evaluate the effect of oocyte source (SLAUG,  $n = 545$  and LOPU,  $n = 423$ ) on the kinetics of IVM (18 vs. 22 vs. 26 h) when submitted to semi defined or defined maturation media. The maturation medium consisted in TCM199 supplemented either with: 1) EGF (10 ng/mL) and cysteamine (100  $\mu$ M) (defined); or 2) EGF (10 ng/mL), 5 IU/mL hCG, 10 IU/mL eCG, 19 ng/mL IGF-1, 2.2 ng/mL FGF, 5  $\mu$ g/mL insulin, 5  $\mu$ g/mL transferrin, 5 ng/mL selenium, 90  $\mu$ g/mL L-cystein, 0.1 mM  $\beta$ -mercaptoethanol, 75  $\mu$ g/mL vitamin C, 720  $\mu$ g/mL glycine, 0.1 mg/mL glutamine and 110  $\mu$ g/mL pyruvate (semi defined). In Experiment 2, we determined the differences on embryo development between both oocyte sources (SLAUG,  $n = 1043$  and LOPU,  $n = 570$ ) when submitted to both media and to either, IVF (Souza *et al.*, 2013, Anim Reprod Sci 138, 82-89) or parthenogenetic activation (PA). Embryos from all groups were vitrified and their viability evaluated after thawing. In Experiment 1, in defined medium, more SLAUG oocytes reached metaphase (M II) stage than LOPU ones at 18 and 22 h ( $P < 0.05$ ). Furthermore, for SLAUG oocytes, M II rate did not change among 18 (87%), 22 (90%) and 26 h (79%), whereas for LOPU oocytes, M II increased significantly ( $P < 0.05$ ) from 53% (18 h) to 72% (22 h), being similar to 26 h (65%). In semi defined medium, no difference in number of oocytes that reached M II stage was observed when oocytes from both origins were matured. These results suggest that the kinetics of IVM is different between oocyte sources and depending on the medium used. In Experiment 2, cleavage rate was significantly higher ( $P < 0.001$ ) after PA than after IVF for all groups. Interestingly, cleavage rate after PA was similar for SLAUG oocytes matured in both media (~90%) whereas it was improved when LOPU oocytes were matured in semi defined (93%) as compared to defined (83%) medium ( $P < 0.05$ ). After IVF, SLAUG oocytes had higher cleavage rate (~67%) as compared to LOPU ones (~39%) ( $P < 0.05$ ), whereas the percentage of blastocysts from cleaved embryos was not different (68 and 67%, respectively). Therefore, SLAUG oocytes developed to blastocyst stage in a greater number than LOPU ones. Vitrified-thawed blastocysts showed similar results in survival (~67%) and hatching (~55%) rates between oocyte sources, maturation media or activation method. In conclusion, SLAUG and LOPU derived oocytes may have different IVM kinetics. Although IVM in goats still need improvement in order to enhance embryo yield, it was possible to generate good quality embryos from LOPU and SLAUG derived oocytes.



A157 OPU-IVP and ET

### **Embryonic development of bovine oocyte matured *in vitro* in the presence of GDF-9 and BMP-15**

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**Keywords:** BMP-15, GDF-9, IVM.

The GDF-9 (Growth Differentiation Factor 9) and 15-BMP (Bone Morphogenetic Protein 15) are growth factors of TGF- $\beta$  family. They are produced by the oocyte, and are involved in regulation of important ovarian functions. Studies have suggested that addition of these factors during maturation improves embryonic development *in vitro*. The aim of this study was to evaluate the effect of GDF-9 and BMP-15 during *in vitro* maturation of bovine oocytes on *in vitro* embryo production. Cumulus Oocyte Complexes (COCs) were aspirated from slaughterhouse ovaries and, after selection COCs classified as I and II were distributed into 4 treatment, performed in 5 replicates: Cont: control oocytes matured in TCM 199 supplemented with FSH (0.01 IU / ml), l-glutamine (0.1 mg / ml), amikacin (0.075 mg / ml) and 0.4% BSA; GDF: oocytes matured in control medium supplemented with GDF-9 (100 ng / ml; R&D systems); BMP: oocytes matured in control medium supplemented with BMP-15 (10 ng/ml; R&D systems), and BMP + GDF: oocytes matured in control medium supplemented with GDF-9 (100 ng / ml) and BMP-15 (10 ng / ml). After maturation, COCs were fertilized and embryos cultured in SOF medium and evaluated on D2, D7 and D8. For statistical analysis, Chi-square test ( $P < .05$ ) was used to compare embryonic development among different treatments. None of the treatments, Cont (n = 134), GDF (n = 128), BMP (n = 130) and BMP + GDF (n = 147) showed differences during embryonic development, when evaluated at D2 (79.1%, 81.3%, 83.8% and 79.6%), D7 (33.6%, 43%, 44.6% and 38.8%) and D8 (36.6%, 43%, 44.6% and 38.8%) respectively. We conclude that the addition of growth factors GDF-9 and BMP-15, alone or in combination, did not influence embryonic *in vitro* development.



A158 OPU-IVP and ET

### Is it possible to increase the efficiency of *in vitro* embryo production in Holstein preselecting donors with more oocyte production?

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**Keywords:** donor, embryos, Holstein.

The commercial availability of sex-sorted semen resulted in a significant increase of *in vitro* embryo production (IVEP) in dairy breeds. Despite the lower oocyte production (Palhão et al., 2011), cleavage rates, and embryo production (Grázia et al., 2012) than in zebu dairy breeds, there is a great interest in the use of IVEP in Holstein. The present study aimed to evaluate whether the selection of donors based on the production of cumulus-oocyte complexes (COC) can compensate the lower IVEP efficiency in this breed. Data from follicular aspiration and *in vitro* cultures performed in Gir (N = 266) and Holstein (N = 270) donors from 2011 to 2013 in the same IVPE laboratory were used. Data were analyzed by ANOVA and differences between groups were compared by Tukey's test. Percentage differences were compared by Chi-square test. The results are shown as mean  $\pm$  SEM. As expected, the average of total COCs production and number of viable COCs were higher in Gir than in Holstein donors (19.1 $\pm$ 0.9 and 11.6 $\pm$ 0.6 vs. 13.3 $\pm$ 0.6 and 6.8 $\pm$ 0.3, respectively; P < 0.0001) and the total embryo production rate was also higher in Gir breed (55.1% $\pm$ 0.01% vs. 36.3% $\pm$ 0.02%). The percentage of IVEP batches with 40 to 100% of embryo production was also higher in Gir (68.7% vs. 39.2%) than in Holstein donors (P < 0.05). In Holstein, correlations between total oocytes recovered or total viable oocyte and embryo production rate per IVEP batch were negative (R = -0.02 and R = -0.05, respectively; P > 0.05). The retrospective analysis in Holstein breed showed that the IVEP batches with results of 0 to 20% and 80 to 100% were associated with aspiration sessions that produced the same number of total and viable COC (10.3 $\pm$ 0.8 and 5.2 $\pm$ 0.5 vs. 12.0 $\pm$ 1.4 and 5.6 $\pm$ 0.7, respectively; P < 0.0001). Coherently, the hypothetical selection of Holstein donors ranked in the first and second quartiles of total oocyte production would still result in a total number of embryos produced and embryo production rate lower than in Gir donors (4.0 $\pm$ 0.3 and 41.0% vs. 6.5 $\pm$ 0.3 and 55.1%, respectively; P < 0.001). These results demonstrate that the selection of donors based on COCs production is not the best strategy to optimize IVEP results in Holstein, and highlight the need to investigate other potential predictive parameters for the system efficiency.



A159 OPU-IVP and ET

### **Investigation on the presence of glucocorticoid receptors in bovine cumulus-oocyte complex, oocyte and embryos produced *in vitro***

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**Keywords:** Glucocorticoid receptor, cumulus-oocyte complex, embryo.

Glucocorticoid Receptors (GR) are transcription factors that reside in the cytoplasm while inactive, and are translocated to the nucleus upon activation by binding to their corresponding hormone (Bosscher & Haegeman, 2009, *Molecular Endocrinology*, 23, 281–291). During research performed in our laboratory, it was observed that the addition of cortisol to the culture medium influenced embryo quality during *in vitro* production, which suggests GRs play an active role during early embryogenesis. Therefore, the objective of this study was to verify the presence of GRs in bovine cumulus-oocyte complexes (COCs), immature and mature oocytes, and embryos produced *in vitro*. For immunocytochemistry analysis, COCs, immature and 22-hour matured oocytes, and embryos at stages 2-4 cells (48h), 8-16 cells (72h), morula (120h) and hatched blastocyst (168 h) were fixed in 4% paraformaldehyde (PFA) for 15 minutes. Fixed samples were permeabilized with 0.25% Triton X-100 for 10 minutes and blocked with 1% BSA and 0.3 M glycine for 30 minutes. They were then incubated overnight in the primary antibody solution (1:50) and then for 1 hour with the secondary antibody (1:250) conjugated with FITC. Nuclei were counterstained with propidium iodide. 15 samples per stage were analyzed. All samples were found to be positive for GR at all of the studied stages (100%), and the corresponding negative controls (without antibody 1°) were found to be negative. GRs were observed to show a more peripheral localization in immature COCs. In other stages studied (matured COCs, immature and matured oocytes, and embryos), GRs were observed to be more diffusely distributed throughout the cytoplasm. Therefore, we conclude GRs are present, display stage specific localization during early embryogenesis, and that this may allow these early stages to respond to cortisol therapy.



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### Different sperm selection methods used for ovine *in vitro* embryo production

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**Keywords:** IVP ovine, sperm gradients, swimn up.

The sperm selection method is one step of *in vitro* embryo production systems and influences the embryo development rates. The aim of this study was to compare the efficiency of different sperm selection methods used in ovine IVP systems in terms of embryo development rates. Methods as Swim up (SW), Mini Optiprep<sup>®</sup> (MO), Mini Percol<sup>®</sup> (MP) and Mini Isolate<sup>®</sup> (MI) were compared in terms of cleavage rates at day 2 and development rates at day 8 (blastocysts/oocytes inseminated). Cumulus oocyte complexes (COCs) were aspirated from ovine ovaries obtained at a local slaughterhouse, selected and matured for 22-24h. The same batch of ovine fresh semen was used in the different sperm selection methods in the experiment. The SW method was performed by layering an aliquot of semen under tris glucose citric acid medium. After 30 min of sperm migration in controlled conditions (39 C), the upper portion was removed and centrifuged at 200G for 5 min. The small volume gradients (MP and MI) were both prepared at 90 and 45%. Semen aliquots were layered over MP and MI and centrifuged at 700G for 5 min. The small volume gradient MO was prepared at 30, 28 and 26% and after semen aliquots were layered it was centrifuged at 900G for 15 min. The sperm pellet was isolated and centrifuged at 700G for 5 min in fertilization media, in all treatments. The *in vitro* matured COCs were randomly distributed in four treatments: SW (n=130), MO (n=152), MP (n=120) and MI (n=110) and inseminated with  $1 \times 10^6$  spz/mL spermatozoa maintained in fertilization media during 18h. Embryo culture was performed during 8 days in SOFaa media with 0.8% BSA in bag system in an atmosphere arrangement of 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub>. Embryo development rates were compared using variance analysis and Duncan test at 5% of significance level (SAS). No differences were observed in the cleavage rates (71%, 76%, 75% and 81%) and development rates at D8 (21%, 24%, 16% and 17%) (P>0,05) for SW, MO, MP and MI respectively. The experiment results showed no influence of the different sperm preparation methods over the embryo development rates found in the ovine *in vitro* embryo production systems.



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## Effect of semen incubation prior to insemination on *in vitro* fertilization rate of bovine oocyte

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**Keywords:** bovine, embryo ivp optimization, spermatozoa.

In several situations, the same mating is used for oocyte insemination of different donors that are submitted to ovum pick up in the same day. The use of the same semen straw for IVF in distinct periods of time could decrease the technique cost, especially when using sexed semen. Therefore, the aim of this study was to evaluate the *in vitro* fertilization rate of bovine oocytes, using sperm doses incubated prior to the IVF procedure. An ejaculate of Nelore bulls (n = 4) was collected and frozen in three fractions: non-sexed (NS) – control group, sexed X (SX) and sexed Y (SY). A fourth group was formed by a pool of X and Y spermatozoa (SXY). Semen from each group/bull was used for IVF of oocytes grades I and II, obtained from slaughterhouse ovaries. One straw of each group/bull was thawed and washed in SOF 700 g/ 5 min. Semen from each treatment was incubated in SOF medium with a final concentration of  $2 \times 10^6$  sperm/mL for 0h, 2h and 4h until the *in vitro* insemination time. Twelve and 18 hours post-insemination (pi), the zygotes (n=2051) were denuded, fixed and stained with Lacmoid for evaluation of *in vitro* fertilization rate. Data were analyzed by ANOVA with Tukey test (P <0.05). There was no difference in fertilization rates between 12 and 18 h pi for groups and incubation period. The NS group ( $63.5 \pm 12.3$ ,  $37.2 \pm 12.4$ ,  $30.8 \pm 12.1$ ) showed higher fertilization rates, compared to the SXY group ( $34.7 \pm 12.2$ ,  $19.5 \pm 10.2$ ,  $8.9 \pm 14.5$ ) at 0, 2 and 4 h pre-incubation periods, respectively. However, both groups showed lower fertilization rate at 2 h and 4 h pre-incubation, compared to 0h. For the groups SX and SY, no significant difference was observed for fertilization rates at 0 h ( $41.5 \pm 12.6$  and  $34.9 \pm 12.2$ ), 2 h ( $22.7 \pm 10.7$  and  $32.8 \pm 12.5$ ) and 4 h ( $11.5 \pm 8.1$  and  $15.8 \pm 9.5$ ). Nevertheless, a decrease in fertilization rate occurred at 2 h pre-incubation in group SX, while in group SY this decrease only was observed when sperm pre-incubated for 4 h were used. Since bovine sperm pre-incubation decreased *in vitro* fertilization rates for both sexed and non-sexed sperm cells, we can conclude that it is not an alternative for IVP optimization. However, sperm containing Y chromosome kept the same fertilization capacity after 2 h of incubation, being probably more resistant in culture conditions than X sperm.



A162 OPU-IVP and ET

### Ovarian follicle stimulation prior to opu and in vitro embryo production in holstein cows

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**Keywords:** bovine, follicle aspiration, FSH.

Due to the growing demand for *in vitro* embryo production (IVEP), researchers have focused in developing strategies to enhance the efficiency of this biotechnique. The goal of this study was to evaluate IVEP in Holstein cows exposed to ovarian follicle stimulation prior ovum pick-up (OPU), as proposed by Nivet et al. (2012, *Reproduction*, v.143, p.165-171). Non-lactating Holstein cows were randomly assigned in a crossover design to the following experimental groups: Control: OPU at a random day of the estrous cycle (n = 35); FSH group: OPU after follicle wave synchronization and superstimulation with FSH (n = 35). At Day 0 of the protocol (which is the random day of the estrous cycle), a norgestomet ear implant (Crestar, Intervet, Brazil) was added and follicles  $\geq 7$  mm in diameter were aspirated for wave synchronization. Treatment with FSH (Folltropin-V, Bioniche Animal Health, Ontario, Canada) started 36 h later (six injections of 40 mg 12 h apart). The OPU was performed 44 h after the last FSH injection. In both groups, all of the follicles  $> 2.5$  mm were aspirated and the oocytes were classified for subsequent IVEP. The rate of viable oocytes was higher in the FSH compared to control group ( $60.0 \pm 3.5$  vs.  $57.1 \pm 3.7\%$ ;  $P = 0.01$ ). The blastocyst rate was also higher in the FSH group ( $18.8 \pm 2.4$  vs.  $13.2 \pm 2.3\%$ ;  $P = 0.09$ ). However, there was no difference in the average total number of oocytes retrieved ( $16.5 \pm 2.0$  vs.  $20.5 \pm 2.4$ ;  $P = 0.16$ ), viable oocytes ( $11.1 \pm 1.5$  vs.  $12.3 \pm 1.7$ ;  $P = 0.13$ ), and embryos ( $3.1 \pm 0.6$  vs.  $2.7 \pm 0.5$ ;  $P = 0.22$ ) per cow per OPU between the FSH and control groups, respectively. Therefore, utilizing the protocol for ovarian follicle stimulation did not improve IVEP results in Holstein cows. These results are indicative that there are no economic advantages of using a protocol of ovarian follicle stimulation prior to OPU for IVEP.

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### **Influence of BSA and/or FBS supplementation during IVM on oocyte lipid droplets and mitochondria behavior, and embryo lipid accumulation in bovines**

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**Keywords:** IVM, lipids, mitochondria.

Lipid oxidation in oocyte is responsible for energy intake and it is related to maturation and embryo development. This study aimed to evaluate the influence of BSA and/or FBS on oocyte Lipid Droplets (LD) and Mitochondria (M) behavior and embryonic LD. Three experiments were designed, and the oocytes underwent IVM with: 8mg/mL BSA (BSA group, GBSA), 10%FBS (GFBS) or 6mg/mL BSA + 5%FBS (GB+F). In the first experiment we evaluated the LD and M migration during IVM by confocal microscopy using fluorimetric techniques (RED Mitotracker CMXRos and LipidTox Green Neutral LipidStain, Molecular Probes, USA). In the second experiment, we studied the size (<2µm, 2 to 6µm or >6µm in diameter) and the amount of LD (by the sum of the area), and the amount of mitochondria (by Fluorescence Intensity-FI, with values between 0 and 255 for each pixel in the selected area) in immature and mature oocytes. In experiments I and II the results were compared to those obtained in *in vivo* matured oocytes (GIV), which were also evaluated for progression to MII by nuclear staining with Hoechst33342 (Molecular Probes). In experiment III, rate of BL and embryonic lipid accumulation was evaluated as mentioned in experiment II. Data from experiment I and the rate of BL were evaluated by X<sup>2</sup> test and the remaining data by Mann Whitney. Regarding LD and M migration from a peripheral localization to a dispersed localization, in experiment I, the groups submitted to IVM failed to achieve the migration rate obtained for GIV (30/58-51.7%). Also, GFBS (21/65-32.3%) had higher rates than GBSA (7/61-11.5%) and GB+F (10/66-15.1%). The groups FBS and B+F showed higher LD migration rates (63/65-96.9%, 63/66-95.4%, respectively) compared to GBSA (44/61-72.1%), whereas in GIV when nuclear mature and immature oocytes were separated, all oocytes in MII (38/58-65%) also showed the same LD migration pattern. In Experiment II, we observed that IVM caused an increase in oocyte lipid content that was not present in GIV (361.7µm<sup>2</sup>; immature oocytes:440.2µm<sup>2</sup>), with no differences in the sizes of droplets between groups. Furthermore, GFBS showed higher LD accumulation (746.9 µm<sup>2</sup>) and a smaller number of M (FI of 11.7) than the GBSA (LD:565.1µm<sup>2</sup> and M:15.71) without differing from GB+F (LD:663.7µm<sup>2</sup> and M:12.82). GIV was the only group able to maintain a correlation between both organelles (r<sup>2</sup> = 0.73). In experiment III, GBSA failed to achieve BL rates seen on GFBS and GB+F (279/1410-19.79%, 336/927-36.25% and 368/1062-34.65%, respectively). There were no differences in embryonic lipid accumulation between groups (GBSA:2526µm<sup>2</sup>, GFBS:2588µm<sup>2</sup> and GB+F:2163µm<sup>2</sup>), however, for droplet size, a higher percentage of LD between 2-6µm (53.3%) was observed in GFBS in comparison to GB+F (43.9%). Therefore, FBS in IVM does not promote greater embryo lipid accumulation than BSA, but it causes differences in comparison to *in vivo* maturation in regards to distribution and quantification of oocyte LD and M.



A164 OPU-IVP and ET

### **Validation of an *in vitro* bovine embryos production program with oocytes and embryos transportation through long distances**

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**Keywords:** *bos indicus*, embryo transfer, sexed semen.

The large territory associated with the distance between bovine production sites and *in vitro* Embryos Production (IVEP) laboratories, for many times have limited the use of this technology in large scale in Brazil (Marinho *et al.*, 2012, Animal Reproduction, 9, 323-328). Thus, the objective of this work was evaluate the viability of an IVEP program in which oocyte maturation and embryo culture occurred partially during transportation, and to determine the effect of the fertilization with sexed semen upon the number of cleaved oocytes, the embryo production and the pregnancy rate of recipients. For this purpose, 123 oocytes from Nelore donors raised in Bahia state were obtained by ultrasound guided follicular aspiration (OPU). These oocytes began their maturation process in cryotubes containing TCM-99 modified bicarbonate, high humidity and 5% of CO<sub>2</sub> in air, and they were submitted to transport inside a portable incubator under 38,5°C temperature to a commercial laboratory in the state of São Paulo. The transportation lasted around 18 and 24 hours and the maturation was finished in the laboratory, followed by *in vitro* fertilization and culture. The fertilization was done with conventional semen (group 1 – CONV) or sexed semen for female (group 2 – SEX). Six days after fertilization, embryos produced were sent to Bahia state, under similar conditions of the oocytes, except by the medium appropriate for embryo culture. Embryos were transferred to recipients in the seventh day after *in vitro* fertilization. Pregnancy diagnosis was done by transrectal ultrasonography 30 days after IVF, using a 5,0 MHz linear transducer. Data were analyzed in *Statistical Package for Social Science* (SPSS, 19<sup>th</sup> version). Mean and standard deviation from variables were obtained by descriptive analysis; differences between number of cleaved structures and embryos produced according to CONV and SEX groups were compared using Student's T test; pregnancy rates between CONV and SEX group were compared using the chi-square (X<sup>2</sup>) test. Embryo rate was 32,85%, with an average 10,09±6,2 of embryos produced/ OPU. Pregnancy rate was 33,12% corresponding to an average of 2,71±1,2 pregnancies/ OPU. The number of cleaved oocytes did not differ (P=0,49) between conventional (CONV=61,49%) or sexed semen (SEX=63,43%). The number of embryos produced was similar (CONV=38,11% vs. SEX=47,76%, P=0,054), as well as pregnancy rate (CONV=33,37% vs. SEX=31,93%, P=0,586). The transportation of oocytes and embryos has been proved to be efficient, even when sexed semen was utilized, showing that IVEP programs are viable when donors and recipients are located far from the production laboratories.



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### **Efficiency of superovulatory treatment started near the time of emergence of the first or last follicular waves of progesterone protocol in Santa Ines ewes during non-breeding season**

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**Keywords:** embryos, follicular wave emergence, superovulation.

This study was designed to investigate if the time of onset of FSH treatment (near the emergence of first or last follicular wave of P4 protocol) influenced the superovulatory response and embryo yield in Santa Ines ewes during non-breeding season (between the months of July to November). Days of emergence of follicular waves were defined in a previous study that evaluated the follicular dynamic during estrus synchronization treatments (Oliveira et al., *Acta Scientiae Veterinariae*, v.40, p.361, 2011). Twenty Santa Ines ewes were submitted to one of two superovulatory protocols according to the time FSH treatments were initiated (G-first wave, n=10 and G-last wave, n=10). On Day 0 all ewes received a P4 device (CIDR®) and injection of 37.5 µg of D-cloprostenol, i.m. The FSH treatments started on Days 4 and 10 of protocol for G-first and G-last, respectively. The superovulatory regimen consisted of eight i.m. injections of pFSH administrated twice daily (40, 40, 30, 30, 20, 20, 10 and 10 mg of pFSH). The P4 device was removed on Day 6 and 12 for G-first and G-last, respectively. At CIDR removal, all ewes received another injection of 37.5 µg of D-cloprostenol and a dose of 200IU of eCG. During four days after the P4 device removal, ewes were mated by a fertile ram. The superovulatory response was evaluated through examination of the ovaries by ultrasonography (three times daily, during the mating period) and laparoscopy (concomitantly the embryo collections). Embryo collections were accomplished 7 days after CIDR withdrawal by laparotomy, and classified according to their development. A sample number of embryos of each treatment were also fixed and stained by TUNEL techniques to assess the apoptotic cells percentage. Data were analyzed by GLIMMIX using SAS. There was no effect between treatments ( $P>0.05$ ) for the superovulatory response (percentage of ovulated follicles:  $89.20\pm 4.15\%$  vs.  $83.50\pm 6.17\%$ ; number of ovulations:  $12.40\pm 0.95$  vs.  $12.60\pm 1.87$ ; number of luteinized unovulated follicles:  $1.70\pm 0.70$  vs.  $3.10\pm 1.59$  for G-first wave and G-last wave, respectively). Similarly, there was no effect ( $P>0.05$ ) on embryos yields (recovery rate:  $69.90\pm 5.61\%$  vs.  $51.70\pm 7.50\%$ ; mean number of structures recovered:  $8.60\pm 1.01$  vs.  $5.90\pm 0.90$ ; number of viable embryos:  $3.20\pm 0.81$  vs.  $1.80\pm 0.80$ ; and viability rate:  $40.50\pm 11.93$  vs.  $32.70\pm 11.74$ , for G-first wave and G-last wave, respectively). Moreover, there was no effect between treatments ( $P>0.05$ ) for the apoptotic cells percentage (G-first wave:  $3.10\pm 1.66\%$  and G-Last wave:  $12.76\pm 4.34\%$ ). In conclusion, there were no differences in superovulatory response and embryo yield between FSH treatments initiated during the first or last follicular waves of progesterone treatment in Santa Ines ewes during non-breeding season.

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A166 OPU-IVP and ET

### **Influence of the number of oocytes obtained per Nelore donor in the *in vitro* embryo production**

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**Keywords:** bovine, IVP, oocyte production.

The aim of the present study was to compare the embryo production among bovine females with high, intermediate and low oocyte production obtained after OPU. Nelore cattle (*Bos indicus*, n = 66, 72-96-month-old) underwent ultrasound-guided follicular aspiration using a 7.5-convex intravaginal array transducer. Briefly, COCs recovered were classified and transported to the laboratory for IVEP. IVF was performed with semen from a bull of known fertility. Cattle were assigned to groups according the oocyte production, as follows: G-High (n = 22,  $\geq 40$  oocytes), G-Intermediate (n = 25, 18-25 oocytes) and G-Low (n = 19,  $\leq 7$  oocytes). Data were analyzed using the Qui-square test ( $P < 0.05$ ). The mean number of COCs recovered was  $50.4 \pm 11.30$  (G-High),  $21.4 \pm 3.04$  (G-Intermediate) and  $5.3 \pm 1.50$  (G-Low,  $P < 0.05$ ). The mean number of viable oocytes was  $40.4 \pm 10.60$  (G-High),  $14.8 \pm 3.02$  (G-Intermediate) and  $3.8 \pm 1.08$  (G-Low,  $P < 0.05$ ) and the proportion of viable oocytes was 80% (888/1109, G-High), 69% (371/534, G-Intermediate) and 71% (72/101, G-Low,  $P < 0.05$ ). Cleavage rates were 79% (762/965, G-High), 74% (348/472, G-Intermediate) and 71% (65/92, G-Low,  $P < 0.05$ ) and blastocyst rates were 42% (405/965, G-High), 32% (153/472, G-Intermediate) and 13% (12/92, G-Low,  $P < 0.05$ ). The mean number of viable embryos was  $18.4 \pm 6.71$  (G-High),  $6.1 \pm 3.57$  (G-Intermediate) and  $0.6 \pm 0.68$  (G-Low,  $P < 0.05$ ) and the percentage of vitrifiable embryos was 81% (329/405, G-High), 77% (118/153, G-Intermediate) and 58% (7/12, G-Low,  $P < 0.05$ ). It is concluded that Nelore cows with high oocyte production had greater percentage of viable oocytes, blastocyst rates and percentage of vitrifiable embryos compared to the cattle with low oocyte production following OPU/IVP. Cattle with high oocyte production produced ~30-fold more viable embryos compared to the low ones. Thus, Nelore cattle with high oocyte production had greater performance after *in vitro* embryo production.



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### Use of cilostamide in pre-maturing of bovine oocytes

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**Keywords:** block, competency, meiosis.

Several substances have been tested in order to block the resumption of meiosis, providing additional time for the acquisition of oocyte competence (Luciano *et al.*, 2011, *Bio. of Reproduction*, 85, 1252-9; Thomas *et al.*, 2004, *Bio. of Reproduction*, 71, 1142-9). However, no increase in embryo production has been observed when oocytes are subjected to meiotic arrest. High concentrations of cAMP are responsible for maintaining the oocyte in germinal vesicle. In this context, the cilostamide, which is an oocyte specific phosphodiesterase type-3, acts directly on the cAMP concentration, and can be an alternative to be used in the meiotic arrest (Vanhoutte *et al.*, 2008, *Mol. Reproduction and Development*, 75, 1021 -30; Thomas *et al.*, 2004, *Bio. of Reproduction*, 71, 1142-9). The present study aimed to evaluate the effect of pre-maturation, in which the meiotic arrest was performed for 8 and 24 hours in the presence of cilostamide (10  $\mu$ m; Sigma®) in IVP of bovine embryos. Cumulus oocyte complexes (COCs) were obtained from slaughterhouse ovaries and, after selection, were divided into 4 groups: C18: control where COCs were matured for 18h (n = 117); C24: control where COCs were matured for 24h (n = 120); PM8: COCs pre-matured for 8 and matured for 18h (n = 112) and PM24: COCs pre-matured for 24 hours and matured for 18h (n = 118). After maturation, COCs from all groups were fertilized and cultured until day 7 (D7) of development. Cleavage and blastocyst rates were evaluated in D2 and D7, respectively. To assess embryo quality only the embryos measuring >160 $\mu$ m on D7 were stained with Hoechst 33342 and used to quantify the nuclei number. Data for the measurement of embryos and nuclei number were analyzed by Kruskal-Wallis test and embryonic development by chi-square test (P <.05). Cleavage and blastocyst rates were higher for C24 (90.8  $\pm$  4.4% and 47.51  $\pm$  3.5%) and PM8 (89.3  $\pm$  6.7% and 44.64  $\pm$  4.2%) compared to C18 groups (76.9  $\pm$  3.6% and 40.17  $\pm$  2.2%) and PM24 (70.3  $\pm$  6.1% and 27.1  $\pm$  2.8%). The percentage of D7 embryos > 160 $\mu$ m, and the nuclei number were lower in PM 24 (53.2% and 105.1  $\pm$  14.3) when compared to others, that were similar (C18: 72.3% and 121.6 $\pm$ 14.3; C24: 70.1% and 127.5 $\pm$ 13.8; PM8: 72.0% and 121.3 $\pm$ 12.8). Results suggest that the addition of cilostamide in pre-maturation for 8h followed by 18h IVM does not impaired embryo development, but had no beneficial effect on embryo yield and quality. Conversely, pre-maturing for 24h followed by 18h IVM had detrimental effect on embryo yield and quality.



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### **In vitro production of bovine embryos in physico substrate borosilicate**

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**Keywords:** borosilicate, IVEP, polystyrene.

Despite the advances obtained in the systems of *in vitro* Embryos Production (IVEP) in cattle, especially in relation to embryo quality, studies about the environment and culture conditions are still necessary. In this context, due the possible leaching of bioactive chemicals components from plastic material (McDonald et al., 2008, Science 322, 917) to the cell culture medium and can be harmful to the embryony development, the aim of this study was to evaluate the influence different physical supports (polystyrene petri dish x borosilicate glass petri dish) in the IVEP. For this purpose, ovaries from abattoir were aspirated and the selected oocytes randomly distributed between two Experimental Groups: *in vitro* maturation, fertilization and embryonic development in Polystyrene Dish (PD) or Borosilicate Dish (BD). The cleavage rates, blastocyst formation, morphological analysis, kinetics of development and total number of cells were subjected to ANOVA (Bonferroni post-test, with significance level of 5%). There was no statistical difference ( $P>0.05$ ) between Groups PD e BD in none of the analyzed criteria: cleavage rates ( $80\pm 16$  and  $72\pm 14.4\%$ , respectively), blastocyst formation ( $40\pm 8$  and  $37\pm 7.4\%$ , respectively), total number of cells ( $102\pm 39.4$  and  $84\pm 28.4$ , respectively), qualitative morphological analysis – classified as Grade 1, 2 and 3 (PD:  $45.2\pm 12.7$ ;  $29.9\pm 18.5$ ;  $17.1\pm 4.0$  e BD:  $48.9\pm 5.6$ ;  $29.8\pm 3.6$ ;  $21.3\pm 5.9$ , respectively) and kinetics of development (PD:  $15.0\pm 0.8$ ;  $22.5\pm 0.8$ ;  $35\pm 0.4$  e  $27.5\pm 0.4$  e BD:  $18.9\pm 0.5$ ;  $29.7\pm 0.8$ ;  $29.7\pm 1.1$  e  $21.6\pm 0.5$ , respectively to Early Blastocyst, Blastocyst, Expanded Blastocyst and Hatched Blastocyst). In conclusion there is no difference on the type of physical support used in IVEP, although more studies are necessary, since only the morphological evaluation can't be enough for qualitative diagnosis embryo.



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### **Variables associated with pregnancy rate in embryo recipient mares**

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**Keywords:** embryo, mare, recipient.

The present study aimed to evaluate the possible variables on pregnancy rate in a program of embryo transfer in mares, in the breeding season of the years 2010, 2011 and 2012. One hundred and seventy-seven mares between three and fifteen years of age, located in the city of Limeira, São Paulo State, were submitted to a gynecological examination on the day of embryo transfer and classified according to the number of births, asynchrony with ovulation of donor, uterine tonus, uterine echogenicity and opening degree of the cervix. The results were submitted to analysis of variance ( $P < 0.05$ ). The pregnancy rate was similar ( $p > 0.05$ ) between nulliparous, primiparous and multiparous mares (84%, 78% and 76%, respectively). The transfers done on the fifth day of the estrous cycle showed an increase in pregnancy rate (87.2%,  $P = 0.05$ ) compared to the pregnancy rate on days four (76.7%), six (61.9%) and seven (66.6%). The pregnancy rate was similar ( $P > 0.05$ ) between recipients with intermediary uterine tonus (75.3%) and firm and tubular tonus (75%). In the ultrasonographic evaluation, recipients with moderately heterogeneous echogenicity and presence of endometrial folds showed a numerical increase (80.5%,  $p = 0.06$ ) in pregnancy rate compared to recipients with heterogeneous echogenicity, presence of endometrial folds (63%) and with homogeneous echogenicity, with no evidence of endometrial folds (72%). The opening degree of the cervix had no influence on the results ( $p > 0.05$ ) (open cervix: 82.3%; partially open cervix: 73% and close cervix: 80%). Although gynecological examination is of fundamental importance, the results showed a greater relevance of asynchrony between donor and recipient.



A170 OPU-IVP and ET

### Low sperm concentration reduces polyspermy and enables the birth of piglets after *in vitro* produced embryo transfer

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**Keywords:** *in vitro* fertilization, pig IVP, semen.

The pig IVP until now is inefficient, with failures in many steps of production. The high rate of polyspermy after IVF is one of the problems. This study aimed to adjust the sperm concentration in IVF of porcine oocytes, allowing obtaining embryos that result in newborns piglets. Follicles 3 to 6 mm in diameter were aspirated from ovaries of prepubertal gilts, obtained at slaughterhouse, and IVM in TCM-199 medium, supplemented with 26,19mM Sodium Bicarbonate, 25% de Follicular Fluid, 0.1mg/mL L-Cysteine, 10ng/mL EGF, 100UI/mL G Penicillin, 0,1mg/mL Streptomycin, 0.5mg/mL LH, 0.01UI/mL de FSH and 1mM de dbcAMP. After 22 h of maturation, oocytes were transferred to a maturation medium free of LH, FSH and dbcAMP for additional 18 to 20 h. After maturation and mechanically denudation, oocytes were selected for the presence of first polar body. Only matured oocytes (n=179) in three replications were submitted to IVF in mTBM fertilization medium supplemented with 0.4mg/mL Caffeine and 2mg/mL BSA. Semen was obtained from fresh ejaculate, and selection of viable spermatozoa was performed by mini-Percoll method. Selected sperm were incubated with oocytes in one of the following concentrations: 62,500, 125,000 or 250,000 sperm/mL. After a 3-h co-incubation period, the excess of spermatozoa adhered to the zone pellucid was mechanically removed, and the presumptive zygotes were fixed to verify the penetration and polyspermy rates. Data was evaluated by Qui Square test with 5% significance level. Higher penetration rates (100.0% 56/56) was observed with 250,000 spermatozoa/mL compared with 62,500 (92.4% 61/66) and 125,000 (89.5% 51/57) which did not differ between them. In the monospermy evaluation, 62,500 spermatozoa concentration showed the higher rate (42.6% 26/61) that was not different from 125,000 spermatozoa concentration (27.5% 14/51), being this one similar to that observed with 250.000 spermatozoa concentration (17.9% 10/56). With basis on the obtained results oocytes (n=319) were IVM and IVF with 62,500 sperm/mL, and cultured in PZM-3 medium supplemented with 3mM/mL BSA in an incubator at 38.8 °C under 5% CO<sub>2</sub> atmosphere. After cleavage evaluation (72.3% 230/319), cleaved zygotes were surgically transferred into one of the oviducts of two previously synchronized gilts (100 and 101 zygotes). Remaining zygotes (n=29) were cultured resulting in 34.5% blastocyst rate. Both the recipients were confirmed pregnant by ultrasound at day 30 of pregnancy. However, only one remained pregnant to term. During parturition there was need for obstetric aid. Six piglets were delivered (5 females and 1 male, with 1,183KG average weight. All six piglets were healthy and had a developmental performance similar to their counterparts. We concluded that *in vitro* fertilization with a reduced sperm concentration (62,500 / mL) reduces the rate of polyspermy in pig oocytes, allowing to obtain embryos that result in the birth of healthy piglets.



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## Luteal vascularization of bovine embryo transfer recipients with color Doppler ultrasonography

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**Keywords:** *corpus luteum*, doppler, progesterone.

Luteal angiogenesis is an active and variable process according to the estrous cycle. Luteal blood vessels are important to an efficient functioning of the corpus luteum (CL) as an endocrine gland (SHIRASUNA et al., J. Reprod. Dev. 56: 124-130, 2010). The CL irrigated area increases as well with a volume and a progesterone plasmatic concentration (ACOSTA et al., Reproduction, 125: 759-767, 2003). The aim of this trial was to evaluate the CL vascularization score of bovine embryo transfer recipients in the transfer day and compare this result with the pregnancy rate. Were used 43 Nelore breed cows who received a vaginal device with 1.0g of progesterone (Sincrogest, Ouro Fino, Brazil) and 2mg estradiol benzoate (Sincrodiol, Ouro Fino, Brazil) intramuscular. Eight days after that the progesterone devices were removed and they received 250µg cloprostenol (Sincrocio, Ouro Fino, Brazil) and 0.3mg estradiol cypionate (E.C.P<sup>®</sup>, Pfizer, Brazil) intramuscular. Seventeen days after the hormonal treatment the cows received *in vitro* produced embryos. The ovaries with CL were evaluated before the embryo transfer by transretal ultrasonography at color Doppler mode (My Lab<sup>®</sup>30 Vet Gold, Esaote, Italy) using a 7.5 MHz scanner probe, recording the images for 30 seconds with the scanner probe positioned in the area which provided the best clearance in the observation of the CL and blood flow. The corpus CL vascularization was scored by a trained technician upon the study of the recorded images with the ultrasound device own program (My LabDesk, Esaote, Italy) and expressed by the percentage of vascularization area by total CL area. The pregnancy diagnosis was made 23 days after the embryo transfer with the same ultrasound device at B mode. The vascularization scores and the pregnancy rates were analyzed by Fisher test and the CL total area by Tukey test (P<0.05%). Among the studied animals was found a minimal value of 40% and a maximum of 70% of vascularization, then four groups were provided between this interval by the vascularization score of 40, 50, 60, 70% and correlated with the pregnancy rate after transfer. The CL total area showed no difference (P>0.05) between the groups 40%, 50%, 60% and 70%, which resulted in 17.51mm, 16.69mm, 16.84mm and 17.56mm, respectively. The group 40% showed 0% (0/12) pregnancy which differed significantly (P<0.05) with the other groups 50, 60, 70% that resulted in the pregnancy rates of 40% (4/10), 45.5% (5/11) and 60% (6/10), similar (P>0.05). The result suggests that the evaluation of the CL functional activity can be more accountancy than the volumetrical evaluation to indicate the recipients suitable for embryo transfer.



A172 OPU-IVP and ET

### **Effect of the use of a chemical regulator of PI3K/AKT pathway in *in vitro* production of bovine embryos**

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**Keywords:** embryo, PI3K/AKT, regulator.

The PI3K/AKT pathway is involved in several cell functions as protein synthesis, cell proliferation and differentiation and microtubule dynamics. This pathway acts by phosphorylation of initiation factors, components of the cell division cycle, transcription factors and proteins involved in microtubule formation and cell adhesion. Recently, it has been reported that this pathway can regulate the meiosis of oocytes in particular the metaphase I/II transition, being part of the MAPK3/1 and MAPK14 cascade in oocyte and cumulus cells in cattle. (Uzbekova et al., 2009, *Reproduction*, 138, 235-246). Thus, the aim of this study was to evaluate the influence of the PI3K/AKT pathway in the early embryo development, when a chemical regulator of this pathway – Wortmannin - is added to the maturation medium in an only concentration (which value is in interval of 5 to 100nM). Thus, we used 210 oocytes from slaughterhouse ovaries and classified as grade I and II. After selection, IVM was performed in drops of 100  $\mu$ L (medium 199 + FBS + antibiotics) in the presence or absence (control) of the regulator, in an incubator at 5% of CO<sub>2</sub>, 38,5°C for 22h. For IVF, sperm were selected by the technique of mini-percoll. The matured COC<sub>s</sub> were transferred to 100 $\mu$ L IVF drops using the fertilizing dose of 2x10<sup>6</sup> spermatozoa/ mL for 18h. After IVF, the presumptive zygotes (10/drop) were cultured for 8 days in 100 $\mu$ L drops (medium 199+ FBS + antibiotics). Evaluation of cleavage and blastocyst rates was done 72h and 8 days after IVF, respectively, and compared using the T Test (LSD) at a 5% significance level. There was no difference in cleavage and blastocyst rate, respectively, between the control group (75.5% and 30.2%) and treated group (76.5% and 38.1%). In conclusion, the use of the PI3K/AKT pathway in regulating the maturation medium in the concentration used in this study did not improve the cleavage and blastocyst rates of bovine embryos produced *in vitro*.



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### **Association of FSH and LH prior to follicular aspiration as a way to increase *in vitro* embryo production in the Gyr breed**

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**Keywords:** Gyr, OPU, PIVE.

The knowledge in physiology associated with hormonal therapies available can improve the results of follicular aspiration and PIVE. The use of FSH and LH can reduce variations quantitative and / or qualitative oocyte recovery by follicular aspiration. The objective was to compare the results of PIVE in zebu donors associating FSH and LH prior to follicular aspiration. In the study, 24 Gyr donors underwent three treatments (three groups, G1, G2 and G3) with an interval of 21 days between them, in a crossover design. In the control group (G1) cows were aspirated without any hormonal intervention in follicular wave, whereas the G2 and G3 were administered FSH (Folltropin, Bioniche) 20mg + LH (Lutropin, Bioniche) 1.25 mg by intramuscularly depth, 48 and 24 h prior to follicular aspiration, respectively. The PIVE was performed in the laboratory of the EMDGA (Station Breeding and Dissemination of Animal Genetics), with semen of the bull same for all groups. The mean oocyte retrieval and embryo production were compared by Tukey test at 5% probability. The number of oocytes (mean  $\pm$  standard deviation) obtained by aspiration was  $19.7 \pm 8.3$ ,  $22.2 \pm 9.5$  and  $21.4 \pm 9$ , and embryos (mean  $\pm$  SD)  $4.1 \pm 2$ ,  $4$ ,  $6.6 \pm 2.5$  and  $6.5 \pm 2.7$  for G1, G2 and G3, respectively, not registering statistical differences between the results obtained for the number of oocytes. In the conditions proposed by the study, the treatments were not efficient to increase the number of oocytes, however G2 and G3 association with FSH and LH before follicular aspiration promoted an improvement in the number of embryos per aspiration. The G2 and G3 showed no significant statistical differences among themselves, indicating little difference between the time intervals practiced.



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### **Effect of meiotic arrest with roscovitine, butyrolactone and this association on *in vitro* production embryos bovine**

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**Keywords:** bovine, meiosis inhibition, oocyte.

One alternative to improve the quality of oocyte and consequently the quality of embryo is the use of drugs that induce inhibition of meiotic maturation (nuclear), causing at the same time a better cytoplasmic maturation. This study, evaluated the effect of the addition of a meiotic arrest roscovitine (ROS) and butyrolactone-I on in vitro production of bovine embryos. Nelore oocytes were matured in TCM-199 with Earle's salt + 10% FCS, FSH and LH, in 5% CO<sub>2</sub> atmosphere. To delay meiosis, the oocytes were maintained for 6 h in medium in presence of Roscovitine (12.5µM), Butyrolactone I (50µM) and Roscovitine (6.25µM + Butyrolactone I (25µM). Then the oocytes were cultured for 18 h in agent-free medium to meiosis resume, completing 24 h of maturation. After 24 h of maturation (day 0), oocytes were fertilized in human tubal fluid (HTF – Irvine, New Zeland) under the same condition above. Semen was selected through Percoll gradient and the concentration adjusted to 2 x 10<sup>6</sup> sperm/mL. The presumably zygotes were culture in 90µL droplets of SOFaa + 0.6% BSA + 2.5% FCS in 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N<sub>2</sub> atmosphere until day 7, when blastocyst rate was evaluated. There were made 5 replicates (200 oocytes/replicate). Data were analyzed with ANOVA, followed by Tukey test using the general linear model (PROC GLM) of SAS 9.2. The level of significance adopted was 5%. No statistical differences were observed in blastocyst production rate: Control: 42.3 ± 2.7%; Roscovitine 12.5µM: 39.6 ± 3.0%; Butyrolactone I 50 µM: 42.2 ± 2.3% and Roscovitine (6.25 µM) + Butyrolactone I (50 µM): 38.0 ± 4.5%. Thus, the addition of inhibitors of meiosis has not compromised embryonic development.

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A175 OPU-IVP and ET

## Evaluation of melatonin antioxidant properties upon *in vitro* bovine embryo production system

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**Keywords:** bovine, reactive oxygen species, tbars.

This study aimed to evaluate melatonin effect over bovine *in vitro* embryo production (IVEP) system. Embryo IVP rates were assessed and *in vitro* embryo culture medium thiobarbituric acid assays (TBARS) were performed. Melatonin is an effective free radical scavenger (Tan et al, 2002, Current Topics in Medical Chemistry 2, 181-197) and beneficial effects of melatonin have been demonstrated in IVP for some species (Tamura et al., 2008, J. Pineal Res. 2008, 44, 280-287). Follicles (2 to 7 mm) were aspirated from slaughterhouse-derived ovaries. Cumulus-oocyte complexes (COCs) who had a compact cumulus and oocyte with homogeneous cytoplasm were selected and randomly allocated (35 to 55 per group) on either control Group (CG) or Melatonin Treatment Group (MG). COC's were *in vitro* matured (IVM) for 22 h in 400µl of TCM199 (Day -1). For *in vitro* fertilization (IVF) COC's were moved to fertilization medium 400µl and were inseminated with frozen-thawed semen previously submitted to discontinuous Percoll gradient and incubated for 18 h (Day 0). After IVF presumptive zygotes were striped from remaining cumulus cells by incubation with hyaluronidase and gentle pipetting, then moved to 400µl of KSOM- BSA (Day 1). On Day 3 embryos were inspected under a stereomicroscope to evaluate cleavage rate, and 400µl KSOM-20% FCS was added. On Day 5 200µl of medium were removed, and kept for TBARS and 400µl KSOM-10% FCS was added. On Day 7 embryos were evaluated regarding their developmental stage, percentages were calculated over the total of presumptive zygotes. On Day 8 200µl of medium were removed, and kept for TBARS. All procedures were performed on a Nunc Multidishes 4 well dish without mineral oil overlay. During IVM and IVC groups received either 0ng/mL or 50 ng/mL of melatonin, CG and MG respectively. TBARS are expressed in nanograms of thiobarbituric reactive substances per mL. Statistical analysis was performed using SAS system for Windows 9.2 parametric data was submitted to student T-test and non parametric to Wilcoxon, presented mean  $\pm$  Standard error and median (1<sup>st</sup>, 3<sup>rd</sup> quartile) respectively. Differences were considered meaningful when  $p < 0.05$ . Melatonin treatment neither influenced cleavage (MG 73.94 $\pm$ 2.23; CG 73.11 $\pm$ 2.46) nor blastocyst rates on Day 7: % initial blastocysts MG 2.27 (0; 2.94) CG 2.47 (1.67; 4.55); % of Blastocysts MG 8.51 (6.15; 12.24) CG 10 (5.88; 9.62); % Expanded blastocysts MG 6.25 (3.03; 10.53) CG 5.96 (3.17; 9.62); % Total Blastocysts MG 19.72 (12.82; 26.53) CG 20.92 (14.67; 28.57). Unexpectedly melatonin treatment increased TBARS levels on medium both at D5, CG (141.7 $\pm$ 6.87); MG (240.4 $\pm$ 9.04), and at D8, CG (204.8 $\pm$ 14.53); MG (268.3 $\pm$ 16.68). Melatonin did not affect bovine *in vitro* embryo production rates as well as antioxidant properties were not demonstrated.



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### Effect of quercetin on glutathione levels in bovine oocyte maturation *in vitro*

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**Keywords:** glutathione, oocytes, quercetin.

Reactive oxygen species (ROS) are produced by physiological metabolism of oocytes and excess can cause deleterious effects on cell function, inducing apoptosis (Yang et al., 1998, Hum Reprod., 13, 998-1002). Glutathione (GSH) is the main molecule of defense against ROS in oocytes (De Matos et al., 1995, Mol Reprod Dev., 42, 432-436). When cumulus-oocyte complexes (COCs) are removed from their natural environment is increased production of ROS in this complex, justifying the use of antioxidant in the technique of *in vitro* production of embryos. Quercetin is a flavonoid and has excelled in scientific circles for their antioxidant (Liu et al., 2010, Toxicol In vitro., 24, 516-522). The aim of this study was to evaluate the effect of quercetin on the levels of intracellular GSH in bovine oocytes matured *in vitro*, compared with oocytes matured in the presence of cysteamine, which is a precursor of GSH. The COCs were aspirated from slaughterhouse ovaries and selected (grade I and II). A group of immature oocytes was used to determine the levels of GSH and the other three groups were subjected to maturation in 100  $\mu$ L drops (TCM 199 with 10% FCS, 5.0 mg / mL LH 0.5 mg / FSH mL, 0.2 mM pyruvate and 50 g / ml gentamicin) supplemented with 2 mM quercetin, 100 mM cysteamine and in the absence of antioxidants (control), for 22 hours at 38.8 ° C 5% CO<sub>2</sub>. Each drop contained about 20 oocytes. To assess the levels of GSH, oocytes were mechanically separated from cumulus cells in PBS and transferred to a 10  $\mu$ L droplet of PBS-PVA containing 50 mM CMF2HC fluorescent probe for 30 minutes at 38.8 ° C. To control technique, a group of matured oocytes were treated with 0.009% H<sub>2</sub>O<sub>2</sub> for 30 minutes and determined the levels of GSH. The fluorescence emission was recorded by a camera Infinity1-1 attached to the microscope Nikon Eclipse Ti with UV-2A filter. The captured images were analyzed using the Infinity V6.2.0 software and fluorescence intensity was converted to arbitrary values in all groups. Statistical analyzes were performed using ANOVA followed by Bonferroni's test ( $p < 0.05$ ). In the experiment we used 210 oocytes, randomly divided into five treatments with three replicates. The intracellular GSH level in immature oocytes was determined as a value (1.00), and was lower ( $P < 0.05$ ) than oocytes matured MIV/H<sub>2</sub>O<sub>2</sub> (1.31) and control (1.67). GSH levels did not differ significantly ( $P > 0.05$ ) between control and quercetin (1.64), but both differed ( $P < 0.05$ ) in the group with cysteamine (1.76). In conclusion, matured bovine oocytes have higher GSH concentration compared to immature oocytes. Additionally, cysteamine increase GSH levels, while quercetin maintained the same level found in the control treatment.



A177 OPU-IVP and ET

### **Effect of mefenamic acid on pregnancy rates in recipient mares on day 10 pos ovulation (d10)**

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**Keywords:** ácido mefenamic, embryo transfer, receptor mares.

An embryo exposed to an asynchronous uterus may be subject to developmental factors and hormone levels that do not correspond to its developmental stage, what may result in changes in the rates of development or embryo death (Barnes, 2000, *Theriogenology*, 53, 649-658). Mares that ovulate after the embryo donor are usually better recipient candidates than those ovulating earlier, particularly if ovulation occurred 2 days before (McKinnon and Voss, 1992, *Equine Reproduction*, 19, 179-185). The pregnancy rate of embryo recipients under natural conditions reduces after day 9 of ovulation, limiting their use thereafter (Whitewashed et al., 2007, *Brazilian Journal of Animal Science*, 36, 360-368). The aim of this study was to investigate the anti-inflammatory effect of mefenamic acid on pregnancy rates after embryo transfer in recipients on day 10 of the estrous cycle (D10). For the production of embryos, donor mares had their cycle monitored by rectal palpation and ultrasonography. When follicles reached approximately 35 mm and endometrial edema was detected, ovulation was induced with 1.0 mg of deslorelin acetate IM and 24 h later the animals were inseminated with 500x10<sup>6</sup> viable spermatozoa previously diluted in the skim milk based dilutor Botu-cum (Biotech- Botucatu SP). Eight days after confirmation of ovulation uterine flush was performed to recover the embryos. A total of 33 animals were used as recipients. They were examined daily by rectal palpation and ultrasonography until the day of ovulation and randomly distributed into two groups: group 1 (n = 18) Control: embryo transfer ten days after ovulation without any treatment; and group 2 (n = 15) embryo transfer ten days after ovulation, treatmentd with 1 g of mefenamic acid orally manipulated in paste form (Powervet handling veterinary, SP-Brazil) on the day of transfer and for two more days. The transcervical transfer technique was used and only grade 1 and 2 embryos (McKinnon and Squires, 1988, *J. Am Vet Med. Ass*, 192, 401-406) were transferred. Pregnancy diagnosis was performed six days after ET. We used the Fisher exact test for analysis of the percentages of gestation between groups, which did not differ (P  $\square$  0.05). The pregnancy rate in the group treated with mefenamic acid was 33.3% (05/15) and in the control group 33.3% (06/18). Thus, it can be inferred in this experiment, that there was no effect of the treatment of mefenamic acid on pregnancy rates in recipients D10.



A178 OPU-IVP and ET

### Characterization of individual differences in the efficiency of superovulation in the Gyr breed

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**Keywords:** follicular growth, FSH, zebu.

Most of the previous studies aiming to improve superovulation evaluated only differences in the number of corpora lutea (CL) observed and of embryos recovered. Therefore, differences in total follicular population before hormonal stimulation and in response parameters associated to follicle growth were frequently neglected, causing bias in the conclusion. The aim of the present study was to characterize individual variation in both relative and absolute efficiency of superovulation, based on ovarian follicular population. Non-lactating Gyr breed cows (n=2) and heifers (n=15), kept under the same management, were used. Follicle growth was synchronized (D0) using an intravaginal progesterone device and an injection of estradiol benzoate. Superovulations started on D5 with the injection of 200 UI FSHp, following a conventional protocol. The number and diameter of the follicles present before (D4) and during superovulation (D5 to D8), as well as the number of CL at flushing (D16), were evaluated by ultrasonography. Follicular population was ranked according to size ( $\leq 4$  mm, 5-7 mm,  $\geq 8$  mm), and changes in the percentage of follicles in each size class were used to calculate relative efficiency. The absolute efficiency was determined by the ratio number of embryos recovered: number of follicles  $\leq 4$  mm on D5. Results are shown as mean $\pm$ SEM. As expected, there was a great individual variation in the superovulation outcomes, both considering the number of CL (0 to 28, mean 12.6 $\pm$ 2.1; CV=68.2%) and embryos collected (0 to 15, mean 5.1 $\pm$ 1.1; CV=92.4%). There was no increase ( $P>0.05$ ) in total follicular population during treatment, and the correlation between the number of follicles during superovulation and the further number of CL or embryos remained relatively constant between D5 and D8 ( $r=0.56$  to  $0.65$  and  $r=0.70$  to  $0.79$ , respectively;  $P<0.01$ ). FSH treatment induced a progressive ( $P<0.05$ ) but partial mobilization of small follicles to larger size classes. The relative efficiency of the follicle growth stimulation was 41.9 $\pm$ 5.5% (0 to 75.6%), and this was the endpoint with the largest correlation ( $R=0.80$ ;  $P<0.0001$ ) with the absolute efficiency of the process (12.2 $\pm$ 2.1%, ranging from 0.0 to 25.0%). Retrospective analysis demonstrated that donors with relative efficiency  $>50\%$  had a number of follicles  $\leq 4$  mm on D1 similar to those with efficiency  $<50\%$  (41.6 $\pm$ 6.8 vs 42.1 $\pm$ 3.1;  $P<0.001$ ), but produced more CL and embryos (17.8 $\pm$ 2.5 and 7.6 $\pm$ 1.7 vs 6.9 $\pm$ 2.1 and 2.4 $\pm$ 0.9; respectively,  $P<0.001$ ). In conclusion, individual differences in follicular population and in the follicle response to FSH are important sources of variation in superovulation results, and shall be taken into account for experimental design.

**Acknowledgments:** FAPEMIG, CAPES, Biotran LTDA.



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### **Bovine oocyte transportation in environment with or without control gas atmosphere**

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**Keywords:** gas atmosphere, IVP, maturation.

Maturation stage includes all events that enable maximum expression of oocyte potential for development after fertilization. Indeed, it is one of the most important phases of IVP because in this period the oocyte reaches the ability to complete the next events (IVF and IVC). Several factors such as nutrients, atmosphere, temperature and pH are important and should be controlled during *in vitro* production targeting to simulate the characteristics of *in vivo* follicular environment. The aim of this study was to evaluate the effect of time (1, 8 and 24h) and of oocytes transportation device (with and without automatic control of gaseous atmosphere) on *in vitro* embryo production. Cumulus-oocyte complexes (COCs) were obtained from slaughterhouse ovaries, selected (only grades 1, 2 and 3), and grouped in 15 - 20 structures in cryotubes with TCM 199 supplemented with bicarbonate plus LH, FSH, estradiol, sodium pyruvate and fetal calf serum (FCS). Then, cryotubes were filled with a gas mixture containing 5% CO<sub>2</sub>, 5% O<sub>2</sub> established in N<sub>2</sub>, and placed in two different transportation devices: with controlled temperature and gaseous atmosphere (L1, Carrier Lab Mix Touch, WTA Watanabe Applied Technology Ltda EPP, Cravinhos, Brazil) and only with control of temperature (L2, Carrier oocytes MOD toi-16i, WTA Watanabe Applied Technology Ltda EPP, Cravinhos, Brazil). Cryotubes were kept on transportation devices for 1, 8 and 24 hours (T1, T2 e T3, respectively). A total of 679 viable oocytes were assigned into 6 treatments in 8 replicates. After the exposure period, COCs were transferred to IVM medium and cultured at 38 °C and 5% CO<sub>2</sub> until they completed the 24-hour period of maturation. The embryo production was assessed on the seventh (D7) and tenth (D10) days after *in vitro* fertilization. The experiment was arranged in 3 x 2 factorial design (time x carrier). Data were analyzed by GLM (general linear models), with means compared by Student Newman-Keuls (SAS), considering the effects of time, transportation device and interaction. There was no effect ( $P > 0.05$ ) of transportation device (L1 and L2) on the cleavage rate ( $68.3 \pm 3.1$  and  $71.7 \pm 2.5\%$ , respectively), blastocysts production on D7 ( $26.9 \pm 2.6$  and  $29.4 \pm 2.4\%$ , respectively) and on D10 ( $31.1 \pm 2.5$  and  $31.0 \pm 2.5\%$ , respectively). Similarly, there was no effect of time (1, 8 and 24 hours) on the cleavage rate ( $74.1 \pm 3.4$ ,  $67.2 \pm 3.3$  and  $68.7 \pm 3.7\%$ , respectively), blastocysts on D7 ( $26.8 \pm 2.9$ ,  $31.4 \pm 3.0$  and  $26.2 \pm 3.4\%$ , respectively) or on D10 ( $29.6 \pm 3.1$ ,  $35.2 \pm 2.6$  and  $28.2 \pm 3.4\%$ , respectively), or interaction between time and transportation device ( $P > 0.05$ ). In conclusion, bovine oocytes can be transported in incubators with or without automatic control of the gaseous environment by extended period of time without affecting the viability of the oocytes or embryonic development potential.

**Acknowledgments:** Fapemig.



A180 OPU-IVP and ET

### **Comparison of recovery rates and pregnancy rates of embryos from quarter horses mares of different ages**

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**Keywords:** age, embryo transfer, mare.

The objective of this study was to evaluate the rate of embryo recovery, embryo quality, and pregnancy rate in mares of different ages. The study was made in two farms located in Limeira/SP, in breeding seasons 2011/2012 and 2012/2013. Were analyzed 342 uterine flushings of Quarter Horse mares (n=162). The procedures divided into three groups according to age, mares from 2 to 7 years (G1, n = 191), 8-17 years (G2, n = 99) and > 18 years (G3, n = 52). Donors with follicles of 35 mm and significant uterine edema were treated with 1 mg of deslorelin or 1250 IU of hCG. The mares were inseminated with cooled semen 24h after ovulation induction and examined 24 h later to verify the occurrence of ovulation. Was used the semen of 25 stallions at a dose of 500 million to 2 billion sperm with progressive motility. Uterine flushing were collected seven to nine days after ovulation and embryo transfer was performed in healthy mares, between the fifth and ninth days of the estrous cycle, with a closed cervix, uterus with homogeneous echogenic, and content without edema on ultrasound evaluation. Pregnancy diagnosis was performed at 15 days of gestation by transrectal ultrasonography. Results were compared by analysis of variance (P <0.05). The results were grouped because there was no effect of location on the variables analyzed. The recovery rate of embryos was similar (P <0.05) between groups G1: 71.2% (136/191); G2: 66.6% (66/99); G3: 61.5% (32/52). The stage of embryonic development was similar between groups resulting in 87.5% (119/136), 77.3% (51/66) and 93.8% (30/32) of expanded blastocysts; 2.9% (4/136), 7.6% (5/66) and zero blastocysts; and 9.6% (13/136), 15.2% (10/66) and 6.3% (2/32) of morulae, respectively for G1, G2 and G3. There was a predominance of embryos of excellent quality among the groups (G1: 91.9%; G2: 97.0% e G3: 84.4%). Pregnancy rates were similar between groups G1: 80.9% (110/136); G2: 87.9% (58/66); G3: 68.8% (22/32). Pregnancy per embryo collection was higher (P = 0.05) between donors of G1: 57.6% (110/191) and G2: 58.6% (58/99) relative to G3: 42.3% (22/52). From these results, we observed an effect of age on the reproductive efficiency in large scale embryo transfer.



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### Effect of metabolic state in oocytes competence from nulliparous and multiparous crossbred dairy cows

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**Keywords:** follicular aspiration, heifers, reproductive efficiency.

Infertility in dairy cows is a multifactorial problem, since it depends on the climate, the management, the herd characteristics, and the physiological status of the animals (Westwood et al. 2002, Journal of Dairy Science, 64, 1153-69). The aim of this study was to analyze the effect of metabolic profile in the nuclear maturation rate of crossbred dairy cows (Holstein/Gyr), with 19.0 kg/day milk production. The study consisted of 3 groups: Group 1: Nul - (n = 30) nulliparous cows; Group 2: Mult < 45 (n = 30), multiparous cows during 30 - 45 days postpartum and Group 3: Mult > 45 (n = 30) - multiparous cows during 60 - 90 days postpartum. Oocytes were recovered by ultrasound-guided follicle aspiration and were classified as viable (grade I, II and III) and degenerate (without cumulus, expanded, atresic and degenerate). The viable oocytes were matured in vitro and after 22 h, and were analyzed for the emission of polar body in confocal microscopy. Blood samples of the coccygeal artery/vein and follicular fluid (FF) of the dominant follicle were collected. The following biochemical analyzes were performed: glucose (GLU),  $\beta$ -hydroxybutyrate (BHBA), serum gamaglutamiltransferase (GGT), sodium (Na), potassium (K) and calcium (Ca). Data were analyzed by BioStat 5.0 software. The average number of oocytes recovered was 7.8 (Group 1), 5.8 (Group 2) and 7.4 (Group 3). In groups 1, 2 and 3, an average of 5.0, 2.9 and 3.6 of viable oocytes were recovered, respectively, whereas in these same groups an average of 2.8, 2.8 and 3.7 were classified as degenerated. No significant difference was found ( $P > 0.05$ ) in quality of oocytes between groups, while the nuclear maturation rate was higher in group 1 when compared to groups 2 and 3 ( $P < 0.05$ ). When serum metabolites were analyzed, the rate of GLI was higher ( $P < 0.05$ ) in group 1 compared to the concentrations of the animals in groups 2 and 3, whereas the concentrations of BHBA and GGT were lower in Group 1 when compared to groups 2 and 3 ( $P < 0.05$ ). The serum levels of Na, K and Ca did not differ significantly between groups. The concentrations of GLI, Na and K of the FF were higher in group 1 when compared with group 2.3 ( $P < 0.05$ ), whereas no significant difference in concentrations of BHBA and Ca of the FF between groups was observed. So, it can be concluded that crossbred nulliparous dairy cows had higher maturation rate, and together with the multiparous cows above 45 days postpartum had better physiological state after analyzing the data.



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### Different Pluset superestimation protocols in Holstein lactating cows submitted to OPU

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**Keywords:** Bovine, FSH, oocytes.

In vitro embryo production (IVEP) can accelerate bovine genetic improvement of superior animals. However, the efficiency of this technique can be limited due to variation in the number and quality of oocytes recovered through follicular aspiration (OPU). Hormonal protocols designed to control follicular emergence and development can minimize these variation (Blondin et al., 2002. Biol. Reprod., 66, 38–43). The objective was to evaluate different synchronization and follicular-stimulation protocols during OPU in Holstein donors. Fifteen lactating Holstein donors were used with average milk production of 28.8 liters/day. All cows were submitted to four treatments before OPU (Crossover design). The OPU was performed with an interval of 21 days by the same veterinarian. At OPU, variables evaluated and compared between treatments were the diameter of follicles and the number of total and viable recovered oocytes. The four treatments were as follows: T1: at day 0 (D0) - Intravaginal P4 device (Ciclovar®-Hertape-Calier, Juatuba, Minas Gerais, Brasil<sup>1</sup>) + 0.15mg of D-Cloprostenol (Veteglan®-Hertape-Calier<sup>1</sup>), day 1 (D1) - 3mg of Estradiol Benzoate EB (Benzoato-HC® Hertape-Calier<sup>1</sup>), day 4 (D4) - single dose of 150UI FSH (Pluset®-Hertape-Calier<sup>1</sup>) and day 7 (D7) – OPU; T2: D0 - P4 device + 0.15mg of D-Cloprostenol, D1 - 3mg of EB, D4 to D6 - 50UI FSH twice a day at 12h intervals, D7 - OPU; T3: D0 - P4 device + 0.15mg of D-Cloprostenol, D1 - 3mg of BE, D4 - 100 IU of FSH twice, D5-150UI FSH twice and D6 - 150UI FSH twice, D7 – OPU; T4: D0 - P4 device + 0.15mg of D-Cloprostenol, D1 - 3mg of EB. Follicles largest than 3 mm were aspirated. The average milk production, follicle size and production of viable and total oocytes were compared among groups by Tukey test, at 5% significance level. No differences were found in milk production between treatments ( $P > 0.05$ ). Animals of T3 had the greatest average of follicular development than other treatments (T1:  $0.41 \pm 0.84$ , T2:  $1.38 \pm 1.27$ , T3:  $3.63 \pm 1.77$  and T4:  $3.58 \pm 1.80$ ;  $P < 0.05$ ). Concerning total oocytes, T1 and T3 had greater number of structures than T4 ( $9.06 \pm 0.33$ ,  $11.86 \pm 2.83$  and  $6.86 \pm 2.16$ , respectively). T3 was the group in which best results of viable oocytes were obtained (T1:  $2.06 \pm 2.61$ , T2:  $5.20 \pm 0.51$ , T3:  $9.40 \pm 4.71$  and T4:  $2.06 \pm 2.61$ , respectively). T4 showed greater number of non-viable oocytes. In conclusion, stimulation with 400 IU FSH in six applications can improve the results OPU in Holstein lactating cows.

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A183 OPU-IVP and ET

### Antioxidant effect of quercetin in reducing ROS in bovine oocytes

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**Keywords:** antioxidant, oocyte, Quercetin.

Reactive oxygen species (ROS) are produced during cellular metabolism like hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). In the *in vitro* production system (IVP) of embryos the concentration of ROS can be increased due to factors such as O<sub>2</sub> voltage, light exposure and oocytes/embryos manipulation, causing cellular damage. Thus, the use of antioxidants in IVP embryos system with high O<sub>2</sub> voltages (~20%) is necessary. The quercetin antioxidant action has high capacity for free radicals eliminating, preventing cellular damage. The objective of this study was to evaluate the quercetin ability to remove hydrogen peroxide bovine oocytes treated with 0.009% H<sub>2</sub>O<sub>2</sub>. The oocytes were obtained from slaughterhouse ovaries and grade I and II were selected. To determine the H<sub>2</sub>O<sub>2</sub> concentration to be used in oocytes a pre-experiment was made with different concentrations (data not shown). To assess the ability of quercetin to remove H<sub>2</sub>O<sub>2</sub>, oocytes (without cumulus cells) were treated with several concentrations of quercetin (0,4, 2, 10 and 50 μM), H<sub>2</sub>O<sub>2</sub> (0.009%) and 10 μM of 2',7'-Dichlorofluorescein diacetate (H2DCFDA - used as an indicator for ROS) for 30 minutes at 38.5 °C. Two control groups were also evaluated: Positive and Negative Control (with and without addition of 0.009% H<sub>2</sub>O<sub>2</sub>, respectively) with 10 μM of H2DCFDA for 30 minutes at 38.5 °C. The fluorescence emission of oocytes was captured by camera Infinity1-1 attached to the microscope Nikon Eclipse Ti with filter B\_2E/C 495 absorption/emission 519, and the images analyzed by Infinity software V6.2.0. Arbitrary fluorescence values were analyzed by ANOVA (BioEstat 5.0) and the Bonferroni test (p <0.05). 240 oocytes were evaluated in three replicates and levels of ROS in negative control group were lower (0.56 ± 0.04) than positive control group (1.69 ± 0.16). However, when oocytes were treated with quercetin (0.4, 2, 10 and 50 μM) a decrease in ROS levels (1.51±0.21, 1.32 ± 0.16, 1.34 ± 0.16, 0.89 ± 0.13, respectively) was observed in comparison to Positive control (1.69 ± 0,16), but remained above levels from Negative control (0.56 ± 0.04). According to results obtained, quercetin was effective in reducing hydrogen peroxide concentrations in immature oocytes due its antioxidant effect, showing that can be used on *in vitro* embryo production.



A184 Embriology, Biology of Development and Physiology of Reproduction

### **Effect of expression of pluripotency markers at blastocyst stage on bovine embryo development during elongation**

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**Keywords:** cattle, embryos, pluripotency.

The development of assisted reproductive technologies has been an important advance on animal production improvement. The use of techniques such as embryo transfer, in vitro fertilization and somatic cell nucleus transfer has contributed to accelerate the multiplication of high valuable animals. However when embryos are produced or manipulated in vitro their developmental potential decreases significantly, this impinges upon the production of viable offspring. The lower quality of in vitro produced embryos compared with in vivo-derived ones is due to changes in the gene expression pattern as a result of the response to the in vitro conditions. In bovine, blastocysts for transfer are selected based on the morphology, this does not reflect their developmental potential since many developmental crucial genes might be aberrantly expressed in embryos with an otherwise normal morphology. In this sense the use of genetic markers could be of remarkable value to select good quality bovine embryos. We propose that the expression of pluripotency markers (Oct4, Sox2 and Nanog) at blastocyst stage will correlate with their development potential during the peri-implantation (elongation) period. For this, in vitro produced grade I blastocysts were split in halves; one of half was selected for gene expression analysis while the other was transferred to recipient cattle. Transferred embryos were recovered at day 17, classified by the elongation stage and used for gene expression analysis (Oct4, Sox2, Nanog, Cdx2 and TP1). More than 65 % of the split embryos generated two viable hemi-embryos with the same ability of in vitro re-expansion, similar cell number as well as homogenous gene expression. From 15 embryos that were transferred, 9 (60 %) were collected with different grades of elongation (1-15 cm). A correlation analysis showed that the expression level of pluripotency markers (Oct4, Sox2 and Nanog) at blastocyst correlates with the expression level of the same genes at the elongation stage, but neither with the expression level of trophoblastic markers (Cdx2 and TP1) nor with embryo length.

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A185 Embriology, Biology of Development and Physiology of Reproduction

### **Follicular dynamics, corpus luteum growth, and regression in buffalo heifers and buffalo cows in the colombian humid tropics**

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**Keywords:** buffaloes, corpus luteum, follicular dynamics.

Buffaloes are a species of growing economic importance worldwide; however, there are few studies on their reproductive physiology, especially under tropical conditions. The aim of the study was to characterize the follicular dynamics and luteal growth and regression pattern of multiparous (MB) and heifer (HB) Murrah buffaloes in Colombian humid tropical conditions (Puerto Salgar, Cundinamarca. Lat. 5° 39.075"N and Long. 74° 34.843"O). The HB had an age of 24.72±1.45 months while the MB had 81.97±31.75 months, 2.7 ±0.8 births and 79.5±16.0 days in milk. The animals grazed on *Brachiaria mutica* pastures, with free access to water and mineral salt. 10 MB and 10 HB were synchronized with progesterone-releasing intravaginal device, application of estradiol benzoate on days 0 and 9 and prostaglandin when the device was removed. However, artificial insemination of animals was not performed. Seventeen animals responded to the protocol and 15 days later, the daily ultrasound monitoring began to determine the number and diameter of the structures present in both ovaries (follicles and corpus luteum). Student's *t* test was used to evaluate differences between means of MB and HB. All data are presented as mean ± standard deviation. The estrous cycle length was 22±4.5 for MB and 22±2.7 days for HB. The follicular growth occurred in one wave (n=1; 5.89%), two waves (n=14; 82.35%) or three waves (n=2; 11.76%). In all animals, the first wave emerged the day after ovulation showing 8.33±2.06 and 10±2.72 follicles in MB and HB, while the second wave started on day 11±2.00 and 10.5±2.82, presenting 8.37±2.26 and 8±1.51 follicles, respectively. The third wave began on 16.21±3.1 showing 6.5±1.7 follicles, happening only in MB. The follicular deviation occurred 3.77±1.89 days after emergence in all waves and in both groups, moment when the largest follicle had a diameter of 9.65±1.62 mm. Likewise, the preovulatory follicle maximum diameter was 17±4.6 and 14±2.9 mm for MB and HB. The maximum diameter of the corpus luteum was 19.58±4.16 mm and 17.74±3.32 mm and, its regression started at 15.22±5.26 and 17.62±1.68 days in MB and HB, respectively. There were no significant differences between groups for all of these variables. These results show that MB and HB have estrous cycles with 1, 2 or 3 follicular waves and that 2 wave cycles are the most common, similar to previously reported by others. Futures studies should provide a better understanding of the follicular development in buffaloes in tropical conditions and for the establishment of reference values of clinical relevance.



A186 Embriology, Biology of Development and Physiology of Reproduction

### The effect of blocking (pro)renin receptor on progesterone synthesis during luteinization in cattle

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**Keywords:** aliskiren, ovulation, prorenin.

The renin-angiotensin system is a target for research in physiology of reproduction. In mammals, a possible role of prorenin, independently of renin was suggested in increasing P4 levels in response to the LH surge, because LH release increases prorenin synthesis, although not renin levels, in the blood plasma and follicular fluid (Itskovitz *et al.*, 1988, Ann. N. Y. Acad. Sci., 541, 179-89). Recently the (pro)renin receptor [which binds to prorenin and renin; (P)RR] has been identified in bovine theca and granulosa cells (Ferreira *et al.*, 2011 JRAAS, 12, 475-82). However, the role of prorenin/(P)RR binding in the peri-ovulatory period remains unclear. The aim of this study was to evaluate the effect of blocking (P)RR in the plasma concentration of P4 during luteinization. Thus, european cows with body score  $\geq 3$  had a new follicular wave induced by hormonal protocol according to Santos *et al.* (2012, JRAAS, 13, 91-8). Cows which reached follicular diameter  $\geq 12$ mm received GnRH (100 mg of gonadorelin acetate, IM) and received randomly an intrafollicular injection of 10 $\mu$ M aliskiren (direct renin/prorenin inhibitor; Novartis, Intermed, Wiesbaden, Germany) diluted in PBS (n=6) or PBS alone (control group, n=4). Intrafollicular injections were guided by ultrasound using a 7.5MHz convex probe. The amount of aliskiren injected was determined according to the volume of the fluid in each follicle, which was estimated by linear regression equation  $V = -685.1 + 120.6D$ , where V is the estimated volume and D is the measured diameter of the follicle (Ferreira *et al.*, 2007, Reproduction, 134, 713-9). After intrafollicular injection, the follicles were monitored by ultrasound at 24, 48 and 72h and blood samples were collected from the jugular vein, at 6 and 8 days after GnRH analogue injection, for P4 immunoassays by electrochemiluminescence (CV 1,23%; sensitivity 0,030ng/ml). Cows that showed a  $\geq 2$ mm reduction in follicular diameter within 24h after treatment were excluded from the study. Ovulation was characterized by the disappearance of the large follicle between two consecutive evaluations followed by corpus luteum detection. The absence of ovulation over a 48h period associated to a decrease in follicle diameter was characterized as follicular atresia. The data from P4 assay in different treatments and days were compared by two-way ANOVA. All animals in the control group (PBS, 4/4) and four cows (4/6) in the aliskiren group (66.66%) ovulated. Considering only the cows that ovulated, the P4 concentrations (ng/ml) were reduced in the aliskiren group (3,89 $\pm$ 0,73) compared to the control group (6,89 $\pm$ 1,56) on day 6 (P<0,05), whereas no difference was detected in P4 concentrations between aliskiren group (6,56 $\pm$ 0,77) and control group (8,64 $\pm$ 1,48; P>0,05) at day 8. In conclusion, prorenin/(P)RR participates positively in P4 synthesis during early luteinization in cattle.



A187 Embriology, Biology of Development and Physiology of Reproduction

### **Flemish: a breed in extinction – reproductive profile of Flemish cattle in Southern Brazil**

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**Keywords:** flemish, follicular growth, progesterone levels.

The understanding of physiological phenomena associated with follicular growth and ovulation is essential for the optimization of reproductive biotechniques and, thus, the productive efficiency of the herd (Baruselli et al., Rev Bras Reprod Anim, 31, 205-211). Such biotechniques may determine the reproductive success in threatened species or breeds and, therefore, their maintenance. The introduction of specialized bovine breeds in our herds lead to a gradual disinterest on the Flemish breed, resulting in a drastic decrease in the herd size, with approximately only 50 animals remaining of such breed in the EPAGRI Research Station in Lages, Southern Brazil (Zago et al., Acta Scientiae Veterinariae, 38, 2, 770). The objective of the present study was to describe the behavior of the dominant follicle and variation of plasma progesterone during the estrous cycle of Flemish cows, comparing them to Holstein cows that served as control group. Two groups of female non-lactating pubertal cows, aged from 4 to 6 years-old, of Flemish (FLE, n=5) and Holstein (HOL, n=4) breeds, had the estrus synchronized with two doses of 500 µg sodium Cloprostenol (Sincrocio®, Ouro Fino, Cravinhos, Brasil) IM, at 14 day intervals. As they manifested estrus, the animals had their ovaries evaluated by transrectal ultrasound (M5Vet®, Mindray, Shenzhen, China) every 24 hours, until detection of the second ovulation. The dominant follicle (DF) of each follicular wave was identified, measured and its diameter registered considering the day of the cycle. Blood samples were obtained from each animal, with at intervals of 5 days between collections, until the 20th day of the cycle, and the correspondent plasma was submitted to radioimmunoassay for determination of progesterone plasma levels. For data analysis ANOVA followed by *t* test and Tukey were used. Progesterone plasma concentrations did not differ between groups in none of the evaluated periods ( $P>0,05$ ), reaching minimum levels (mean±SEM) of 0,155±0,016ng/ml for FLE and 0,300±0,048ng/ml for HOL on estrus and maximum levels of 6,651±1,868ng/ml and 5,957±1,233ng/ml on diestrus, respectively, for groups FLE and HOL. The maximum diameter (mean±SEM) of the dominant anovulatory follicles was 12,96±0,52mm for FLE and 12,63±0,65mm for HOL ( $P>0,05$ ). The ovulatory follicles showed maximum diameter of 13,20±0,44mm and 14,68±0,85mm, respectively ( $P>0,05$ ). The dominant follicles of Flemish cows had a day mean growth of 1,237±0,093mm, while for HOL group this measure was 1,172±0,173mm ( $P>0,05$ ). It is concluded that Flemish cows have follicular growth and progesterone plasma profile similar to Holstein cows.



A188 Embriology, Biology of Development and Physiology of Reproduction

### **Dickkopf 1 (DKK1), a canonical WNT signaling inhibitor, promotes development of the trophoctoderm cell lineage in bovine blastocysts**

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**Keywords:** bovine embryo, cell differentiation, DKK1.

Activation of canonical WNT signaling in bovine embryos at day 5-post insemination in vitro impairs blastocyst development and decreases cell number, particularly of trophoctoderm (TE) cells. Deleterious effects of canonical WNT activation can be rescued by DKK1 (Denicol et al., 2013 Scientific Reports, 3, 1–7). Moreover, transfer of embryos exposed to DKK1 from day 5 to 7 increased pregnancy success at day 32 of gestation in lactating Holstein cows (Denicol et al., abstract ID 282728 – Society for the Study of Reproduction, 2013 meeting). The objective of this study was to determine the effect of inhibition of canonical WNT signaling by DKK1 during the morula-to-blastocyst transition on development of TE cells. Embryos were produced in vitro from slaughterhouse-derived oocytes. Maturation time was 20–22 hours and fertilization, 8–10 hours. Both processes took place under 38.5°C and 5% CO<sub>2</sub>. Putative zygotes were randomly allocated in groups of 25 to 30. Culture conditions were 38.5°C, 5% O<sub>2</sub> and 5% CO<sub>2</sub>. On day 5 after insemination, embryos were treated with vehicle (DPBS/BSA 0.1%) or 100 ng/ml human recombinant DKK1. Blastocyst development was evaluated on days 7 and 8. Blastocysts were harvested on both days for staining with immunofluorescence-labeled antibodies. Briefly, embryos were fixed, permeabilized and incubated with a blocking solution. Immunofluorescence was accomplished by sequential incubation with primary and secondary, FITC-conjugated antibody, for detection of CDX2+ nuclei. Hoescht 33342 was used for staining of all cell nuclei. Embryos were observed with a 40x objective using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Gottingen, Germany) and Zeiss filter set 02 (DAPI) and Zeiss filter set 03 (FITC). Digital images were acquired using AxioVision software (Zeiss) and a high-resolution black and white Zeiss AxioCam MRm digital camera. Cell count was performed using ImageJ (National Institutes of Health). The GLM procedure of SAS® (version 9.3; Cary, NC) was used for data analysis. Replicate was considered a random effect. Cells were counted in 125 embryos and 3 replicates. DKK1 did not affect the proportion of blastocysts that were expanded or hatched on either day 7 or 8 ( $P = 0.22$ ). DKK1-treated blastocysts had fewer cells on day 7 ( $107.7 \pm 4.9$  vs  $125.9 \pm 5.7$ ) but there was no difference on day 8 ( $123.7 \pm 4.4$  vs  $122.2 \pm 5.7$ ;  $P = 0.08$ ; treatment x day:  $P = 0.02$ ). Exposure of embryos to DKK1 increased the percent of cells that were TE on both days 7 ( $71.9 \pm 1.8$  vs  $61.6 \pm 2.1$ ) and day 8 ( $73.8 \pm 1.6$  vs  $67.6 \pm 2.1$ ) ( $P < 0.05$ ). In conclusion, DKK1 promotes development of cells of the TE lineage without promoting overall cell proliferation.

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A189 Embriology, Biology of Development and Physiology of Reproduction

### **Comparison of uterine vascularization by Doppler ultrasound of pregnant and not-pregnant cows submitted to FTAI**

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**Keywords:** color doppler, resistance index, uterus.

The aim of this work was to study the uterine vascularization by Doppler ultrasound of inseminated cows, verifying if there is any difference between the resistance index values (RI) and the uterine vascularization score (VS) after AI of cows that became pregnant and the ones not-pregnant. Nelore cows (n=182) were used between 50 and 70 days postpartum assessed by Doppler ultrasonography (Mindray, M5Vet), in spectral (RI) and color Doppler (VS, 0 to 4) modes in three different moments: 30 hours before AI, 4 and 24 hours after AI. The spectral mode was used to objective (RI) evaluation of blood flow of uterine arteries. The localization of uterine arteries was done according to Bollwein et al. (2000, *Theriogenology*, 57, 2053–2061). Color mode was used for subjective evaluation (VS) of the uterine vascularization, being the uterine horns scanned and movies recorded. After that, the images were analyzed by two different examiners. The mean of two examiners was used for statistical analyses. The pregnancy diagnosis was done 30 days after AI, with 99 cows being pregnant (54.39%). The data of pregnant group (n=99) and not-pregnant group (n=83) were analyzed by PROC MIXED of SAS (9.3) and a level of significance of 5%. As there was no interaction between pregnancy diagnosis and time of evaluation (RI: p=0.97; VS: p=0.95), the data was studied according to diagnosis, independent of time and according to time, independent of diagnosis. No differences was observed for RI (p=0.51) and uterine VS (p=0.39) of cows that became pregnant (RI=0.68±0.01; VS=2.36±0.05) and the not-pregnant (RI=0.67±0.01; VS=2.36±0.05). Differences were noted between times of evaluation (RI: p<0.001; VS: p<0.001). Vascularization was higher 4 hours after AI (RI=0.63±0.01; VS=2.61±0.06), the second highest value of vascularization was observed 24 hours after AI (RI=0.67±0.01; VS=2.31±0.05) and the values of 30 hours before AI were lower (RI=0.74±0.01; VS=2.04±0.06). Is not possible to identify changes in the uterine vascularization after AI in cows according to pregnant or not-pregnant status; however, it is possible to note vascular alterations in the uterus during the different periods of the estrous cycle.

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A190 Embriology, Biology of Development and Physiology of Reproduction

### **Presence of conceptus modulates expression of interferon-tau stimulated genes in peripheral blood immune cells of pregnant and non-pregnant beef cows**

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**Keywords:** interferon-tau, monocytes, pregnancy.

Interferon-tau secretion by the pre-implantation conceptus is crucial for corpus luteum maintenance and establishment of pregnancy. This cytokine stimulates the expression of specific genes (ISGs) in peripheral blood mononuclear cells (PBMCs) in dairy cows and sheep. This evidence may serve as the basis for the development of molecular techniques for early pregnancy diagnosis. Ultimately, such approaches may become helpful tools to reduce the interval between inseminations and to optimize beef cattle production. The aim of this study was: (1) to evaluate ISGs transcripts abundance during early pregnancy in beef cows; and (2) to determine the feasibility of detecting non-pregnant cows based on ISGs expression between days 12 and 20 post-AI. Nelore cows (n=27) were submitted to TAI. All animals were treated with estradiol benzoate (2 mg) and received a P4-releasing device (1 g). After 8 days, the devices were removed and the animals received a PGF injection (0.5 mg). Ovulation was induced by GnRH treatment (10 µg) and animals were inseminated 48 hours after P4 device removal. Blood samples were collected from jugular vein on days 12, 15, 20, 22, 30, 45 and 60 post-AI for PBMC isolation by Ficoll® (GE Healthcare) gradient. Pregnancy was diagnosed by ultrasonography on days 25, 30, 45 and 60 post-AI; 10 pregnant animals were detected on day 25 post-AI and one pregnancy was lost between days 45 and 60. Isolated PBMCs from pregnant and non-pregnant cows (n=6/group) were used for RNA extraction and cDNA synthesis. The abundance of the ISGs 2'-5'-oligoadenylate synthetase 1 (OAS-1) and myxovirus resistance 2 (Mx-2) was measured by qPCR. Cyclophilin was used as housekeeping gene for data normalization. Repeated variables were analyzed by split-plot ANOVA using the PROC MIXED procedure (Version 9.2; SAS Institute). For OAS-1 and Mx-2 gene expression, an effect of group, day and their interaction were detected (P<0.05). Expression of OAS-1 and Mx-2 genes in PBMCs progressively increased from day 15 post-AI, reached a peak at day 20 and decreased sharply until day 22 and progressively until day 45 in pregnant cows. Gene expression did not differ across time in non-pregnant cows. On the peak of ISGs expression (day 20 post-AI), the lowest values for relative expression of OAS-1 and Mx-2 were 0.29 and 0.05, respectively. In non-pregnant cows the relative expression of ISGs was never higher than these values. In conclusion, the expression of OAS-1 and Mx-2 genes in PBMCs is stimulated between days 18 and 30 post-AI, and the greater abundance of ISGs on day 20 post-AI may be used for the development of technologies to diagnose early gestation in beef cattle.

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A191 Embriology, Biology of Development and Physiology of Reproduction

### Oocyte quality in post-partum of 3/4 and 7/8 Holstein x Zebu primiparous crossbred cows

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**Keywords:** crosses, oocyte, ovum pick-up.

Crossbred Holstein x Zebu cows are the basis of milk production in Brazil, representing about 75% of the milked cows. In post-partum these cows undergo a period of negative energy balance (NEB), as it occurs in Holstein cows, which causes delay in the return to ovarian activity, associated with decreased oocyte quality. The present study aimed to evaluate oocyte quality in first 57 days post-partum in crossbred cows. Holstein x Zebu primiparous cows 3/4 crossbred (n=13) and 7/8 crossbred (n=14) were used, which were fed a corn silage and concentrate based diet. Ovum pick-ups (OPU) were performed, in average, on 16, 32, 43 and 57 days post-partum, after follicular wave synchronization by puncture of follicles larger than 6 mm, 72 hours before OPU. Oocytes recovered in PBS medium (supplemented with 0.05% fetal calf serum and 20 IU/mL heparin) were classified as viable (grades I, II and III) or not viable and used for *in vitro* embryo production. The variables oocyte total number, viable oocytes and not viable oocytes were analyzed by ANOVA (PROC GLM), while oocyte rate was evaluated by logistic regression (PROC LOGISTIC). In both cases the effects of cross, post-partum days and their interaction were considered. Analyses were performed using SAS software, v.9.2. Crossbred 3/4 cows produced less ( $P < 0.05$ ) total oocytes, viable and not viable oocytes than the crossbred 7/8 cows, that were respectively,  $3.52 \pm 0.39$ ,  $2.44 \pm 0.30$  and  $1.06 \pm 0.20$  in 3/4 crossbred cows, and  $8.43 \pm 0.94$ ,  $6.48 \pm 0.79$  and  $1.93 \pm 0.27$  in 7/8 crossbred cows. In contrast, there was no effect ( $P > 0.05$ ) of post-partum day on the rate of viable oocytes, which was 74.89% (516/689). Also there was no effect ( $P > 0.05$ ) of post-partum day on oocyte production. On average,  $4.89 \pm 0.87$ ,  $4.36 \pm 0.82$ ,  $4.36 \pm 0.86$  and  $4.82 \pm 1.28$  viable oocytes were produced after 16, 32, 43 and 57 days postpartum, respectively. No interaction between the cross and the post-partum days was detected. The lack of influence of days post-partum on the production of oocytes suggests a similar energy balance pattern between groups, which possibly indicates that differences observed between the cows groups is probably due more to individual variation of donors used than to a difference in the cross itself. The lower milk production of crossbred cows compared to Holstein cows, suggests that the intensity and duration of negative energy balance are reduced in crossbred animals, so that during early post-partum (57 days) variation in production and oocyte viability was not observed. However, further studies using a larger number of animals are necessary to establish the relationship between energy balance and oocyte quality in crossbred cows.



A192 Embriology, Biology of Development and Physiology of Reproduction

### Characterization of the histone H3R26me2 modification during *in vitro* development of bovine embryos

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**Keywords:** embryo development, epigenetics, IVP.

The *in vitro* production (IVP) of bovine embryos is a biotechnology of great economic impact for use on genetically superior animals, resulting in greater reproductive efficiency. However, despite several technological advancements, the developmental rates of IVP embryos are much lower than those for *in vivo* embryos. *In vitro* culture is thought to cause adverse effects on embryo development and subsequent gestation. Epigenetics, the regulation of gene expression without changing the DNA sequence, is essential for proper embryo development and may be affected by *in vitro* culture. One of these epigenetic events is the remodeling of histone proteins which are responsible for DNA conformation and required for proper embryonic development. The aim of this study was to evaluate the modification of histone H3R26me2 during pre-implantation development of IVP bovine embryos cultured with and without serum. After *in vitro* maturation and fertilization, bovine embryos were cultured with either 0 or 2.5% serum. Embryos were collected at 2-cell, 4-cell, 8-cell, 16-cell, morula and blastocyst stages from both groups and fixed in 4% paraformaldehyde. Fixed embryos were then used from immunofluorescence utilizing an antibody for H3R26me2. Images of stained embryos were analyzed as a percentage of total DNA. Levels of H3R26me2 changed for both groups over development. In the group cultured in 0% serum, the greatest amount of H3R26me2 staining was at the 4-cell ( $P<0.01$ ), 16-cell ( $P<0.05$ ) and morula ( $P<0.05$ ) stages. In the 2.5% serum group, only 4-cell stage embryos were significantly higher than all other stages ( $P<0.01$ ). These results suggest that the modification of histone H3R26me2 is regulated during development of pre-implantation bovine embryos, and that culture conditions greatly alter this regulation.



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### Supplementation with sunflower seed increases the conception rate in recipients of *in vitro* produced bovine embryos

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**Keywords:** embryonic mortality, linoleic acid, PGF2 $\alpha$ .

Embryo mortality between 15 and 19 days of pregnancy in cattle is apparently due to increase in endometrial PGF2 $\alpha$  release, resulting in the corpus luteum regression. The PGF2 $\alpha$  synthesis may be inhibited in animals supplemented with linoleic acid-rich compounds, among them a sunflower seed. In previous study (Peres et al., 2008, *Acta Scientiae Veterinariae*, 36, 639), Nelore cows supplemented with sunflower seed for 22 days starting at the day of timed AI (TAI) had a higher conception rate (66.7% vs. 46.3%;  $p = 0.02$ ). This study aimed to evaluate the effect of supplementation with sunflower seed on conception rates in embryo recipients. Crossbred heifers received an intravaginal device containing progesterone (1g; CRONIPRESS®; Biogenesis Bago) associated with an intramuscular (IM) injection of estradiol benzoate (2mg; BIOESTROGIN®; Biogenesis Bago). The devices were removed eight days after, when heifers were treated with D-cloprostenol (150 $\mu$ g; CRONIBEN®; Bago Biogenesis) and eCG (400IU; Folligon®, Intervet), both IM. Heifers were treated IM with estradiol benzoate (1mg; BIOESTROGIN®; Biogenesis Bago) 24 hours after device removal. Two days after the removal of the device (D0), it was expected to occur the estrus. Heifers were split into two groups to receive 1.7 kg /animal/day of the following treatments: 40% soybean meal 44% crude protein (CP) and 60% sunflower seed (Sunflower Group;  $n = 106$ ) or 53% of soybean meal with 44% CP and 47% corn (Control Group;  $n = 111$ ). Both supplements were balanced with 72% TDN and 24% CP. Supplements were given for 22 days from D-2 to D19. In D7, *in vitro* produced embryos, Holstein (13.82%) and Nelore (86.18%) breed, were transferred to the recipient by FTET. Pregnancy diagnosis was performed by ultrasonography on D30. Blood samples were collected at D-2, D7 and D19 for measurement of plasma progesterone (P4), total cholesterol, triglycerides, HDL and LDL. Data were analyzed using the SAS procure GLIMMIX. The conception rate was greater ( $p < 0.01$ ) in the Sunflower Group (55.66%; 59/106) than in the Control (36.94%; 41/111). The P4 concentration did not differ ( $P > 0.05$ ) on D7 between the Control ( $4.92 \pm 0.24$  ng/mL) and Sunflower ( $4.72 \pm 0.23$  ng/mL) groups. Greater concentrations of total cholesterol were observed in the Control Group compared to Sunflower on D7 ( $306.02 \pm 11.61$  vs.  $277.10 \pm 11.88$  mg/dL, respectively;  $p < 0.05$ ) and D19 ( $260.51 \pm 7.98$  vs.  $231.95 \pm 7.95$  mg/dL, respectively;  $p < 0.01$ ). Greater concentrations of HDL cholesterol were observed in the Control Group compared to the Sunflower on D7 ( $166.82 \pm 5.88$  vs.  $139.43 \pm 5.74$  mg/dL respectively;  $p < 0.01$ ) and D19 ( $162.01 \pm 4.67$  vs.  $135.57 \pm 4.76$  mg/dL respectively;  $p < 0.01$ ). Supplementation with sunflower seed increases the conception rate in recipients of *in vitro* produced embryos.

**Acknowledgments:** FAPESP, FUNDUNESP e Centre of Recipients Retorno.



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### **Effect of recombinant somatotropin (rbST) on the superovulatory response and early embryo development in bovine (*Bos taurus*)**

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**Keywords:** bovine, embryo, superovulation.

In dairy cows, the fertility rates have shown a decrease in proportion to the increase in milk production in the last 30 to 40 years. This is a continuous trend that occurs at an annual rate of 0.45% in the USA and 1.0% in the UK. Due to this situation, extensive research has been carried out in the last decades in order to enhance fertility in dairy cattle. Recombinant somatotropin treatment at the moment of the artificial insemination has shown a positive effect over pregnancy rates in dairy cows. Nonetheless, the mechanisms by which this effect is held remain to be determined. The objective of this research was to evaluate the effect of rbST on the superovulatory response, ovulation rate, number of recovered oocytes, fertilization rate, stage of embryo development and embryo quality of bovine donors. Female donors of Holstein (16), Brown Swiss (16) and Simmental (16) breeds (n=48), between 2 to 7 years old, in adequate body condition score (2.5 to 3.5) and >100 days in milk, without any diagnosed reproductive disorder were used. The cows were kept in an intensive management system with 50% concentrate and 50% forage. Superovulation was performed according to the following protocol: day 0 DIB insertion (1.0 g progesterone), 1 mg estradiol benzoate (EB), 40 mg coprostenol (PGF2 $\alpha$ ) and 50 mg injectable progesterone; from day 4, 300 mg FSH (Folltropin V, Bioniche Animal Health, Canada), divided in 8 decreasing doses were administered, day 7 DIB withdrawal (Syntex SA, Argentina) and administration of 40 mg cloprostenol, on day 9 artificial insemination (twice, 12 and 24 h post-estrus) and on day 16 collection and evaluation of embryos. The administration of rbST was at first artificial insemination (day 9, AM, n=24). The procedures of collection, washing, recovery and classification of embryos were carried out under standards of the IETS Manual, 2010. The ovulation rate (n of corpora lutea, day 16) and the number of recovered oocytes were not statistically different (p>0.05) between control and rbST groups. However, the quantity of viable embryos (4.2 vs 6.1), proportion of embryos at blastocyst stage – stage 5, 6 and 7 (25 vs 55%), rate of freezable embryos (70% vs 83%) and rate of embryos of excellent quality – grade 1 (42 vs 75%) showed statistical significance (p < 0.05) between control group vs rbST group. In conclusion, the administration of recombinant somatotropin in superovulated cows at the first insemination improved embryo quality and sped up early embryo development.



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### Body morphometry related to gender in *Trachemys scripta elegans* turtles (WIED, 1839)

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**Keywords:** body morphometry, testudine, turtle.

*Trachemys scripta elegans* is an exotic underwater turtle, introduced in Brazil with no proper control by authorities. The abandonment in lagoons, rivers and bodies of water threatens the maintenance of the local biological diversity, due to the extinction of native species by competition or genetic extinction by hybridization. Therefore, studies on the biological aspects of this species are necessary to reduce the risks of environmental impact, to help on its population control or as an experimental model. This study aimed at determining the body biometrics of males and females of *T. scripta elegans*, in order to provide information on their biology when away from their natural habitat. Nine males and 33 females had corporal volume (VC, mL), mass (CM, g), length (LCar, cm) and maximum width (WCar, cm) of the carapace; ;length (LPla,cm) and maximum width (WPla,cm) of the plastron; ;body height (Ch) (Malvasioet et al.,1999, *Arq.Brasil.Zootec*, 16, 91-102) and sexual dimorphism index (SDI) (Gibbons & Lovich,1990, *Herpet. Monogr.*, 4, 1-29) determined. Averages were analyzed by using ANOVA followed by Tukey test and simple correlation coefficients (r) were analyzed among the variables (Assistat 7.6 beta). This study was approved by the CEDEP[1]from UNIVASF[2] (Protocol nr 0001/160412). In the population analyzed, the gender ratio was 3.67:1, where 21.0% (N= 9) were male and 79.0% (N = 33) female. The males presented CM (685.0±298.3), VC (691.8±368.5), LCar (16.1±2.6), WCar (12.9±1.7), LPla (14.8±2.2), WPla (9.6±1.1) and Ch (5.6±1.4) lower (P<0.05) than the females (1178.0±362.3; 1164.3±368.0; 19.8±3.0; 15.0±2.9; 18.7±2.5; 12.2±1.6 and 7.3±1.6, respectively). SDI in the population analyzed was 1.72 for CM, 1.68 for VC, 1.23 for LCar, 1.16 for WCar, 1.26 for LPla, 1.27 for WPla and 1.30 for Ch. In males, very high positive correlations (P<0.01) were observed between CM and VC, WCar, LPla and Ch, between VC and WCar and LPla, LCar and WCar, WCar and LPla and between LPla and WPla, while the positive correlations (P<0.05) occurred between CM and LCar, VC and LCar, WPla and Ch, LCar and LPla, WCar and WPla and Ch and between LPla and Ch. On the other hand, in females, very high positive correlation (P<0.01) was observed among all parameters analyzed, except for Ch that presented positive correlation (P<0.05) with VC and CPla. The results lead to the conclusion that, in the population analyzed: 1-females presented average body mass larger than the males, 2-SDI was, in average, 1.23 times larger for females in all parameters analyzed, 3-the body development of the females occurred uniformly among mass, carapace and plastron, while, in males, it was not observed for WPla, CM and LCar and for Ch, LCar and WPla. These data provide scientific support on the reproductive biology of this exotic species

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### Different media for selection of swine oocytes with Brilliant Cresyl Blue – effects on parthenogenetic embryo development

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**Keywords:** Brilliant Cresyl Blue, parthenogenesis, swine oocyte.

Parthenogenetic activation is a crucial step for the establishment of other technologies such as nuclear transfer. However, regarding swine, more effective protocols need to be established to provide best oocyte selection and to increase rates of oocyte parthenogenesis. Considering this, the oocyte selection by the viability dye Brilliant Cresyl Blue (BCB) is a useful tool. The stained oocytes are considered the most suitable for IVM. Within this context, the aim of this study was to compare development rates by parthenogenesis of swine oocytes selected with 13  $\mu$ M of BCB, in a richer medium, called modified Porcine Zygote Medium (PZM-m) and in PBS solution, the most utilized medium. Prior to IVM 1,621 oocytes were incubated for 60 min at 39 °C in different media, with BCB. The oocytes were classified as positive (stained) or negative (no staining), except for group 1 which was washed in PFF and were not incubated, being considered the general control. The oocytes were distributed in the following groups: Porcine Fluid Follicular (PFF) (n=62); PBS-control (PBSc) (n=336); PZM-m control (PZMc) (n=371); PBS BCB positive (PBS+) (n=336); PBS BCB negative (PBS-) (n=161); PZM-m BCB positive (PZM+) (n=293); PZM-m BCB negative (PZM-) (n=90). IVM was performed in NCSU-23m with eCG, hCG, hypotaurine,  $\beta$ -mercaptoethanol, cysteine, EGF, AMP-c and PFF, in the first 24 hours, followed by NCSU-23 without eCG, hCG and AMP-c, for additional 24 hours. The oocytes were parthenogenetically activated with 20  $\mu$ M ionomicyn for 5 minutes prepared in TCM Hepes medium and 2 mM 6-DMAP for 3 hours in PZM-3, the same medium for embryo culture. On day 4, 10 % of fetal calf serum was added to embryo culture. The results were analyzed by Chi-Square using the software Statistix 9.0. The cleavage rate in group PFF was 67.7% (n=42); 45.1% in PBSc (n=139); 52% in PZMc (n=193); 58.6% in PBS+ (n=197); 0% in PBS- (n=0); 55.3% in PZM+ (n=162); 23.3% in PZM- (n=21). The embryo rate on D7 (blastocyst and morulae) was 1.6% in group PFF (n=1); 14.9% in PBSc (n=46); 18.9% in PZMc (n=70); 8.6% in PBS+ (n=29); 0% in PBS- (n=0); 14.7% in PZM+ (n=43); 1.1% in PZM- (n=1). These results showed that cleavage rates in PZM+ were superior ( $p < 0.05$ ) to PBS+ and PBSc. However, this rate was not superior to PFF. The embryo results showed no difference between the groups PZMc, PZM+ and PBSc, which obtained the best rates. The groups PZM+ and PFF had the best cleavage rates. However, parthenogenetic activation did not show influence of the medium. At the same time, the PZM+ oocytes showed better embryo development than PBS+. Nevertheless, more research and experiments are underway to confirm these findings.



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### **Expression of competence genes in cumulus cells from immature and *in vitro* matured cumulus-oocyte complexes (COCs) morphologically classified into different grades**

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**Keywords:** cumulus cells, gene expression, IVM.

Efficiency of oocyte *in vitro* maturation (IVM) is directly associated to the intrinsic quality of oocyte from the ovarian follicle. Oocytes undergoing IVM are classified according to their morphological characteristics in grades I, II and III, however, little is known about the expression of developmental competence related genes in COCs of different quality grades. The objective of this study was to assess the expression of cumulus cells (CC) competence genes in immature and *in vitro* matured COCs classified into grades I, II and III (Khurana & Niemann, 2000; n=3 replicates/group). Therefore, bovine COCs from 3-8 mm follicles were obtained from slaughterhouse ovaries and separated into three groups according to their morphological classification: grade I (GI, oocytes having an homogeneous, evenly granulated cytoplasm surrounded by a compact CC with more than three layers), grade II (GII, oocytes having an homogeneous evenly granulated cytoplasm with fewer than three CC layers) and grade III (GIII, partially denuded oocytes). The CC from 20 COCs were separated by successive pipetting before (Immature group) and after IVM with bicarbonate TCM199 supplemented with 6% BSA, pyruvate (11 µg/µL), amikacin (16.67 mg/uL), FSH (0.1 mg/mL, Pluset®, Serovet, Rome, Italy), LH (50 mg/mL, Lutropin®, Bioniche, Belleville, Ontario, Canada) and estradiol (1 ug/uL). Total RNA was extracted by RNeasy® kit (Qiagen) and 100ng of RNA was reverse transcribed by SuperScript III® enzyme (Life Technologies). Expression of amphiregulin (AREG), FSH receptor (FSHR) and progesterone receptor (PGR) mRNA was investigated by real-time PCR with StepOnePlus® (Life Technologies) using PowerSybrGreen® (Life Technologies) reagent. Relative mRNA quantification was calculated using  $\Delta\Delta C_t$  method normalized by cyclophilin (CYC-A) as housekeeping gene. Effects of COC grade and time of maturation were tested by ANOVA and groups were compared by Tukey-Kramer HSD test. Differences were considered significant when  $P < 0.05$ . The results showed effect of maturation for all target genes. Expression of PGR and AREG mRNA was higher in matured CC compared to the immature, regardless of morphological degree. In contrast, FSHR mRNA expression decreased with IVM. No differences were found for AREG and FSHR mRNA among grades I, II and III in immature or matured CC, assessed separately. PGR mRNA expression was higher in GI CC compared to GII CC when *in vitro* matured. We conclude that IVM influences expression of competence genes in CC and the regulation of PGR mRNA in matured CC suggests that immature morphologically fittest COCs have better ability to support *in vitro* development. Furthermore, the decreased gene expression of FSHR suggests downregulation of the receptor, possibly associated with IVM inefficiency.

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## Production rates of *in vitro* produced bovine embryos with different developmental kinetics

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**Keywords:** kinetic, quick embryo, slow embryo.

The quiet (or slow) embryo hypothesis mentions that embryo viability is associated with slower metabolism than with faster metabolism, since slow embryos give off less energy to correct genome, transcriptome and proteome damages (Brison et al., 2004, Hum Reproduction, 19, 2319-2324; Leese et al., 2007, Human Reproduction, 22, 3047-3050). However, since *in vitro* produced bovine embryos are cultured in groups, the efficiency of blastocyst formation from slow and fast embryos is not known. In this sense, the objective of this study was to evaluate the rates of cleavage and of conversion to blastocyst in fast and slow *in vitro* produced bovine embryos. In addition, kinetics and standards of embryonic/secretion and consumption of metabolites, by evaluating the culture media by Raman spectroscopy and gas chromatography coupled with mass spectrometry (GC/MS), will be correlated in the future. To evaluate the cleavage rates, the cumulus-oocyte complexes were aspirated from slaughterhouse ovaries, selected (grade 1 and 2), placed in 90  $\mu$ l IVM drops (M-199 + HEPES (20 oocytes / drop) and cultured for 22 hours at 38,5°C and 5% CO<sub>2</sub>. The matured oocytes were *in vitro* fertilized in 90 $\mu$ l droplets of IVF medium (Parrish et al., 1988, Biol. Reprod., 38, 1171-1180) in an atmosphere of 5% CO<sub>2</sub> in air at 38,5°C and high humidity for 18 hours. Subsequently, the zygotes were transferred to individual 20  $\mu$ l culture medium droplets (SOF medium supplemented with essential and nonessential amino acids and 5% fetal bovine serum) in a well of the well system (WOW) (adapted by Feltrin et al., 2006, Proceedings of the 58th Annual Meeting of the SBPC - Florianópolis, SC), and cultured in an incubator with an atmosphere of 5% CO<sub>2</sub> in air and high humidity at 38,5°C for 7 days. Zygotes were classified as fast (4 or more cells) and slow (2-3 cells), according to cleavage rate at 40hpi. Although present, uncleaved embryos after 40hpi were discarded from the analysis. After 168 hours of IVC, embryos were re-evaluated to obtain the blastocyst rates of the fast and slow groups (n=3 replicates). Preliminary data were evaluated by Student's *t* test and there was no difference between the cleavage rates from fast and slow embryos ( $p = 0.1208$ ) with averages of  $28.77 \pm 3.164\%$  for fast embryos and  $34.50 \pm 1.191\%$  for slow embryos. However, differences were observed between the blastocyst rates ( $p = 0.0178$ ) with averages of  $11.6 \pm 1.380\%$  for fast embryos and  $6.4 \pm 1.168\%$  for slow embryos. Therefore, from this study it can be concluded that there is no difference between the number of fast and slow embryos when evaluated at 40hpi. However, there is a higher number of fast embryos reaching blastocyst stage than slow embryos.

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A199 Embriology, Biology of Development and Physiology of Reproduction

### **Different periovulatory endocrine profiles and its relation with spatial distribution of transcripts in the reproductive tract of beef cows**

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**Keywords:** cattle, steroids, uterus.

In cattle, fluctuations of progesterone (P4) and estradiol (E2) concentrations modulate endometrial gene expression, histotroph secretion, conceptus development and pregnancy outcome. Our recent RNAseq studies indicated that changes in the periovulatory endocrine milieu associated with the growth and ovulation of different size follicles regulated endometrial gene expression on day 7 of the estrous cycle. Objectives were to study the effect of the uterine horn relative to the ovary containing the corpus luteum (CL) and the region within the uterine horn ipsilateral to the CL on the expression of selected endometrial genes. An additional objective was to verify whether expression was also modulated on the vagina. The follicular growth of multiparous non-lactating Nelore cows was pharmacologically manipulated in order to obtain groups with large (LF/CL; n=11) or small (SF/CL; n=11) preovulatory follicles and corpora lutea (Mesquita F. *Reprod. Fertil. Dev.*, submitted). Cows were slaughtered seven days after the induction of ovulation with GnRH analogue and fragments from mixed portions of contralateral uterine horn, regions of the ipsilateral uterine horn (anterior, middle, posterior), and mixed fragments of vagina were collected. Gene expression assessment was performed by quantitative PCR in six animals/group. Cyclophilin was used as reference gene. Relative expression of target genes were calculated by the delta-delta CT method with correction for efficiency. The SAS PROC MIXED procedure (Version 9.2; SAS Institute) was used for analysis considering the effect of group (LF/LCL and SF/SCL), uterine horns, regions, and their interactions. A probability of  $P \leq 0.05$  indicated significant effects. The LF/LCL group had larger pre-ovulatory follicles and CL and greater E2 concentrations on D0 and P4 concentrations from D3 to D7. The abundance of transcripts coding the progesterone receptor was greater in the SF/CL (50%). The LF/CL group had a greater abundance of transcripts coded by the estrogen receptor (ESR2; 100%), aldo-keto reductase family 1, member C4 (AKR1C4; 71,3%) and serpin peptidase inhibitor, clade A member 14 (Serpin14; 50%). There was an effect of region for the expression of ESR2, AKR1C4 and Serpin14. Specifically, expression of ESR2 was greater in the middle section and the anterior section showed greater expression for AKR1C4 and Serpin14. No significant effects of interactions were detected for any of the target genes. There was no group effect on any of the selected genes expression in the vagina. In conclusion, although regional patterns in the genes expression were detected across the reproductive tract, such patterns were not affected by distinct periovulatory hormonal milieu.



A200 Embriology, Biology of Development and Physiology of Reproduction

### **Gene expression of sirtuin and related genes in different temperatures of *in vitro* maturation of bovine cumulus-oocyte complexes**

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**Keywords:** Gene expression, sirtuin, *in vitro* maturation.

The adaptations that animals suffer due to high environmental temperatures lead to reduction of food intake and endogenous production of heat. In parallel there are many changes in energy metabolism. It is believed that the changes that occur in response to heat stress are mediated by sirtuins. These proteins act as an interrupter between energy metabolism and cell signaling cascades of other physiological processes. SIRT1 regulates gene expression programs in response to the metabolic state of the cell, thus coordinating the metabolic adaptation of the whole organism. The precise mechanisms regulating this process at cellular level are poorly understood. The aim of this study was to evaluate the expression of sirtuins (SIRT 1 e SIRT 2) and correlated genes (Hes1, BCL11, p53) on cumulus-oocyte complexes at different temperatures of *in vitro* maturation. Bovine ovaries were collected at a local slaughterhouse and transported to the laboratory. Only grade I cumulus-oocyte complexes (COCs) were selected. After that, COCs were placed in an incubator with high humidity and 5% CO<sub>2</sub> in air at temperatures of 37 ° C, 38.5 ° C and 40 ° C for 24 h. After maturation oocytes and cumulus cells were separated, and each group was stored in RNAlater solution (Qiagen, Hilden, Germany) at - 20 °C. Each sample contained 40 oocytes or the corresponding amount of cumulus cell. Three replicates of each group were submitted to RT-PCR and the data were normalized using the endogenous gene RPLPO. To identify differences in expression and their statistical significance the data were submitted to ANOVA including in the model the effect of cell and temperature. The averages were compared by Tukey Test at 5%. The results show that, independent of maturation temperature, the cumulus cell presented differences in expression between mature and immature COCs. The genes SIRT 2, BCL11 and p53 increased their expression, while SIRT 1 showed a reduction of expression after maturation. The only exception was the Hes1, which did not significantly alter the pattern of expression in any situation. In oocytes, the single gene that significantly changed the expression pattern was SIRT1, which presented similar expression between immature (0,048) and oocytes matured at 40° C (0,068) and different expression in oocytes matured at 37 and 38,5° C (0,075; 0,088). Although there was no significant difference among the temperatures of maturation, the oocytes matured at 40 ° C showed the lowest expression among matured (37° C = 0,075; 38,5° C = 0,088; 40° C = 0,068), which allows us to speculate about the reduced expression of this gene when COCs undergo maturation temperatures above 38.5 ° C. Other experiments are being conducted to explain the correlation between genes and their expression in oocytes and cumulus cells.

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A201 Embriology, Biology of Development and Physiology of Reproduction

### **Use of preimplantation genetic diagnosis (PGD) in equine embryo sexing**

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**Keywords:** equine, preimplantation genetic diagnosis, sexing.

The preimplantation genetic diagnosis (PGD) is the removal of a small number of cells from an early development embryo for genetic analysis. In horses, despite PGD presenting enormous potential for determining the sex of the embryos before transfer, the progress for establishment of genetic diagnosis techniques has been slow. In the present study, we investigated new methods based on human reproductive techniques for PGD to improve the efficiency of embryo sexing in horses. Twelve expanded blastocysts were biopsied in Syngro® Holding medium (Bioniche) using a laser (Hamilton Thorne, USA) and a beveled injection pipette (15 µm diameter) coupled to a Narishige micromanipulator. Biopsy pipettes were used to puncture the zona pellucida and the capsule in the region opposite to the inner cell mass followed by aspiration of 10 to 30 trophoblast cells adjacent to the region of the opening of the zona pellucida. The cells were then transferred to microcentrifuge tubes, which were centrifuged at 11.000xg for 10 minutes and stored at -20 °C for future genetic analysis. The WGA (Whole Genome Amplification) method was performed based on fragmentation of genomic DNA followed by isothermal amplification and PCR cycles. The samples were incubated at 50°C for 1 h and heated to 99°C with Single Cell Lysis & Fragmentation Buffer. Then universal oligonucleotide primers were used to amplify the DNA fragment with 25 cycles of PCR. PCR products were loaded on a 2% agarose gel containing ethidium bromide and electrophoresed for 2 h at 60 V or 1 h at 110 V. Results were visualized and photographed using UV light transillumination. The amplified DNA was initially evaluated for sex by PCR for the *SRY* gene, which generated a fragment of 131bp. Because in this test, absence of the male *SRY* signal could occur due to failure of the PCR, the amplified DNA was also evaluated for sex by a duplex PCR for the gene for RNA-binding motif protein, Y-linked (*RBMY* – fragment of 225bp), which is Y-chromosome specific, and glioma pathogenesis-related protein 1 (*GLIPR1* – fragment of 113bp), an autosomal gene. Specificity of the primers was confirmed during a blinded study of 6 blood samples collected from mares and stallions, and the sex was correctly determined in all samples. The sex of the embryos could be detected in 10/12 embryos (83.3%), of which 7 were females and 3 were males. The time required for the confirmation of sexing was approximately 8 hours. All embryos re-expanded after the procedure. The biotechnology here described is simple and fast providing the equine vet a suitable alternative for clinical use.



A202 Embriology, Biology of Development and Physiology of Reproduction

### The antioxidant system in preterm and term canine neonates

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**Keywords:** antioxidante, canine neonates, lung.

With the onset of pulmonary respiration, an exponential increase in the production of free radicals occurs. In order to counteract such event, the development of an antioxidant defense capable of neutralizing the oxidative stress cytotoxicity is necessary. The aim of this study was to compare the development of the antioxidant system, vitality and gas exchange in canine term and preterm neonates. According to the gestational age, 15 neonates were allocated into: Term Group (63 days of gestation,  $n = 5$ ), Premature 57 Group (57 days of gestation,  $n = 5$ ) and Premature 55 Group (55 days of gestation,  $n = 5$ ). Gestational age was assessed by identifying the LH surge through maternal progesterone assay. The pregnant females were submitted to cesarean section and, after the hysterectomy and removal of fetuses from the uterine cavity, the amniotic fluid was collected to assess the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPX), as well as the marker of oxidative stress (TBARS). The quantification of GPX activity was performed by NADPH consumption, SOD through the reduction of cytochrome c by superoxide anion, and TBARS by means of malondialdehyde (MDA) concentration. From neonates, the Apgar score of vitality and body temperature were assessed at birth and after 2 and 4 hours of birth, as well as blood samples were drawn for hemogasometric and SOD, GPX and TBARS evaluation. The experimental design was approved by the Ethics Committee of FMVZ/ USP. Data were compared by ANOVA and Tukey test ( $p \leq 0.5$ ). Four neonates of the Premature 55 Group died within the first hour of life, due to extreme degree of prematurity. There was no statistical difference between groups or among time points for SOD, GPX and TBARS both in serum and in the amniotic fluid. At birth, the Term Group had the highest Apgar score ( $3.8 \pm 0.4$ ), statistically superior to Premature 55 Group ( $2.5 \pm 0.2$ ), but not different from Premature 57 Group. The Term Group reached the satisfactory Apgar score within 2hs of birth ( $8.4 \pm 0.7$ ), while the Premature 57 Group remained low within the first 4 hours of birth ( $5.3 \pm 0.7$ ). There was a progressive increase in body temperature in Term Group during the 4 hours of birth, inferior to Premature 57 Group, with no statistical difference between the times of evaluation. All puppies had blood acidosis, except at 4h for Term Group. Moreover, the Term Group showed higher  $pCO_2$  at birth ( $76 \pm 7.84$ ) compared to Premature 57 ( $47.7 \pm 3.1$ ) and Premature 55 ( $56.7 \pm 3.2$ ) groups. In conclusion, our results suggest that the neonatal antioxidant status does not change according to prematurity, with the same antioxidant ability between premature and term neonates. Puppies have mixed acidosis at birth, but only term neonates can efficiently reverse such imbalance, compared to premature neonates.



A203 Embriology, Biology of Development and Physiology of Reproduction

### Body condition and return of ovarian activity in lactating woolless sheep

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**Keywords:** Leptin, postpartum, progesterone.

The return of cyclic ovarian activity postpartum (RCOA) in small ruminants depends on hormonal interactions in the hypothalamic-pituitary-ovary axis during lactation. However, in early lactation, the pituitary hormones are more directed towards the synthesis and secretion of milk than for the restoration of cyclic ovarian activity, resulting in a period of postpartum anestrus (Rodrigues et al., 2011, Arq Bras. Zootec Vet Med., 63, 171-179). The nutritional management and body condition of lactating ewes directly influence the ovarian activity postpartum and plasma concentrations of leptin, a hormone important for promoting follicular growth and ovulation (Zieba et al., 2008, Can. J. Physiol. Pharm., 59, 7-18). The aim of this study was to investigate the effects of supplementation and body condition of ewes on ovarian activity postpartum. We used 24 ewes belonging to the Technical School of Bom Jesus-PI. During the latter half of pregnancy and 75 days of lactation the sheep had access to pasture grass *Andropogon gayanus* and were randomly divided into two groups according the levels of concentrate supplementation (0.5% and 1.5% of body weight) based on 70% corn bran, 25% soybean meal, 5% vitamin and mineral supplement. Weight and body condition score (BCS) were weekly measured (Thompson and Meyer, 1994, Oregon State, 4). Blood samples were collected twice weekly for determination of progesterone and leptin, performed by radioimmunoassay (Laboratory of Endocrinology, UNESP, Araçatuba, SP) using commercial kits (Coat A Count Progesterone Kit, Siemens®, Multi-Species Leptin RIA kit, Millipore®). The RCOA was considered when progesterone concentrations were greater than 1.0 ng/mL for more than ten days (Minton *et al.*, 2001, J. Reprod. Fertil., 69, 314-320). The percentage of RCOA until the end of lactation was compared by Chi-square between the levels of supplementation and between the classes of BCS (1.0-2.0 and 2.5-3.5). To test the effect of supplementation level and the class of BCS on the plasma concentrations of leptin ANOVA was performed and means were compared by Tukey test. During lactation, 13 animals showed BCS average between 1.0-2.0 and 11 animals showed BCS average between 2.5-3.5. The level of supplementation had no effect on the RCOA and on plasma concentrations of leptin ( $p>0.05$ ). There were no interaction effects of supplementation and the class of BCS ( $p>0.05$ ). The RCOA rate was higher in the group of animals with BCS between 2.5-3.5 than in the group of animals with BCS between 1.0-2.0 (91,9 vs. 61,5%,  $p<0.05$ ), with effect of BCS categories ( $p<0.05$ ) in the RCOA ( $p<0.05$ ). The sheep with BCS between 2.5-3.5 also had a higher concentration of leptin than those with BCS between 1.0-2.0 (0.80 vs. 0.60 ng/ml;  $p<0.05$ ). It is concluded that the body condition of lactating sheep influences the return of cyclic ovarian activity postpartum independently of the levels of supplementation studied in this work.



A204 Embriology, Biology of Development and Physiology of Reproduction

### The ovine perinatal period: an uterine and umbilical blood flow survey

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**Keywords:** gestation, ovine, vascularization.

The pulsed-wave Doppler is a non-invasive diagnostic tool valuable for accurate prenatal follow-up. The present study aimed to evaluate the blood flow of the uterine (UA) and umbilical (UMA) arteries in pregnant ewes from 60 days onwards, during the pharmacological induction of parturition, as well as the uterine blood flow during the puerperal period. Fifteen Santa Inês ewes were used for Doppler ultrasound analysis. Uterine arteries were scanned through a rectal linear probe (5-10 MHz) after the identification of the iliac vessel ramification. Subsequently, the pulsed-wave Doppler was employed to classify the flow velocity waveforms and to automatically calculate the hemodynamic parameters (RI, PI, S/D). In order to localize the umbilical arteries, the abdomen was scanned. A total of nine stable waves were obtained for each artery to calculate the average for each variable. During gestation, ultrasonographic exam was performed at 60, 90 and 120 days. On the 135<sup>th</sup> day of pregnancy, ewes were subjected to lambing induction with a double injection of 0.33 mL/kg (IM) of the antiprogestagen aglepristone (Alizin, Virbac, Brasil), at a 24h interval. During parturition induction, UAs and UMA were evaluated at 3 time-points: 12hs previous to the first injection (M1) and 12hs after each aglepristone injections (M2 and M3). The onset of lambing occurred 49.8±4.9 hours after the first injection of aglepristone. The UAs were also examined at 1, 3, 5, 7, 15 and 30 days post-partum. During gestation and parturition induction, the overall uterine vascularization remained constant, with a low resistance pattern (RI: 60d-0.58±0.01; 90d-0.59±0.02; 120d-0.6±0.01; M1-0.61±0.02), characterized as continuous and high blood flow in the fetal unit. The UMA hemodynamic indexes progressively decreased along gestation (RI: 60d-0.99±0.02; 90d-0.77±0.01; 120d-0.62±0.02). There was no negative influence of the parturition induction on uterine vascular flow, since the UAs hemodynamic indexes remained unchanged after treatment (RI: M2-0.6±0.02; M3-0.6±0.02), simultaneously with the increase of UMA blood flow. Conversely, there was a decrease in UMA resistance after the induction of lambing (RI: M1-0.61±0.02; M2-0.58±0.01; M3-0.55±0.02), suggesting vasodilation to insure fetal oxygenation during parturition. On the other hand, the vascular indexes immediately post-partum were enhanced (RI: M3-0.6±0.02; 1d-0.77±0.02), compared to the results obtained at lambing. Based on these results, we can infer that the mechanism that triggers ovine parturition along with the steroidogenic change have no direct influence on uterine vascular dilation capable of impairing placental perfusion. Therefore, there was a reduction in uterine vascular flow soon after the removal of the vasodilation stimuli. Hence, the uterine blood flow maintains regardless of the increase in umbilical hemodynamic as a manner to guarantee the adequate vascular flow for fetal survival.



A205 Embriology, Biology of Development and Physiology of Reproduction

### **Correlation between maternal body condition and follicular population in the development of bovine fetal gonads from Nelore breed**

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**Keywords:** carcass yield, fetal ovaries, fetal testis.

The cattle body condition reflects energy intake variation, influencing reproductive function and during gestation, promoting direct and indirect effects on fetal growth, including ovary and testicle characteristics. The aim of this study was to correlate maternal carcass weight (CW), carcass yield grade (CYG, scale 1 to 5), age and antral follicle counts (AFC) with weight and volume from fetal gonads in different gestational ages (3 to 8 months). The study used 534 pregnant cows from slaughterhouse located at Araçatuba region and was conducted from June 2012 until March 2013. All cows were distributed to groups according to their AFC as follows: low  $\leq 15$  ( $\geq 2$ mm diameter), intermediate 16 to 30 and high  $\geq 31$  antral follicles. Fetal age was estimated by the formula:  $DG = 8.4 + 0.087C + 5.46\sqrt{L}$ , where DG= Day of gestation and L= fetal length. The measurements from fetal gonads were: height, width, thickness (cm) and weight (g). Data were analyzed by Pearson correlation test (r) and values with  $p < 0.05$  were submitted to non linear regression test. Negative correlation was observed between maternal AFC and fetal testicular weight, and volume ( $r = 0.3$ ,  $p < 0.0001$  and  $r = 0.23$ ,  $p = 0.0014$ ). A positive correlation was observed between maternal CW and fetal weight ovary on the 5<sup>th</sup> and 7<sup>th</sup> months of gestation ( $r = 0.47$ ,  $p = 0.0192$  and  $r = 0.43$ ,  $p = 0.0223$ ). Cattle CYG was positively correlated to fetal ovary weight on the 5<sup>th</sup> month ( $r = 0.55$  and  $p = 0.0057$ ). On the other hand, it was negatively correlated with fetal testicular volume on the 8<sup>th</sup> month ( $r = -0.69$  and  $p = 0.0126$ ). The correlation between maternal age and fetal testicular volume was negative on the 5<sup>th</sup> and 8<sup>th</sup> months ( $r = -0.35$ ,  $p = 0.0353$  and  $r = -0.62$ ,  $p = 0.0334$ ). There was not influence of maternal CYG on fetal length during the gestation. It is known that nutrition affects LH plasma concentrations, which could be a possible explanation for the increase on fetal ovarian weight found on the 5<sup>th</sup> and 7<sup>th</sup> months of gestation. On this period, fetal ovaries begin to exhibit sensitivity of antral follicles to gonadotropin concentrations. To demonstrate this effect, maternal and fetal LH concentrations will be quantified in the next phase of this study. Testicular volume from male fetuses may decrease as consequence from high maternal testosterone concentrations (T), since this can cause a negative feedback on LH secretions by fetal pituitary decreasing testicular growth due to low fetal T concentrations. Collaborating with this assumption, in obese cows high androgen concentrations in the blood and reduction on Leydig cells population in the testes of their male fetuses were observed. Quantification of T and LH concentrations will be held at the next stage of this work, as well as Leydig cells counting, both to prove these assumptions.



A206 Embriology, Biology of Development and Physiology of Reproduction

### **Corpus luteum development and function after supplementation of long-acting progesterone during the early luteal phase in beef cattle**

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**Keywords:** Doppler, estrous cycle, luteolysis.

Progesterone (P4) is essential for establishment and maintenance of pregnancy in mammals. Supplementation of P4 within the first week post artificial insemination has embryotrophic effects in cattle (Carter, *Reprod Fertil*, 20, 368-75). Paradoxically, there is a reduction in corpus luteum (CL) function when exogenous P4 is given on the days next to ovulation indicating a possible interference with development of the CL (Garret, *Prostaglandins*, 36, 85-96). The objective of this study was to evaluate the effects of long-acting P4 supplementation on days 2 or 3 postovulation on CL development and regression in beef cattle. Nelore cows were synchronized with an estradiol/P4-based protocol and treated intramuscularly with a single dose of 150 or 300 mg long-acting P4 (Sincrogest<sup>®</sup>, Ouro Fino Saúde Animal, Brazil) on Day 2 or 3 postovulation (n=6-7 cows/group). Color-Doppler ultrasound scanning and blood sample collection were conducted from Days 2 to 21.5 postovulation. Based on plasma P4 concentrations, the beginning and the end of functional luteolysis were estimated for each cow. Timing of structural luteolysis was estimated based on the area of CL measured in each scanning. Continuous variables were analyzed by split-plot ANOVA using the PROC MIXED procedure (Version 9.2; SAS Institute). Discrete variables related to characteristics of luteolysis were analyzed by one-way ANOVA. Mean comparisons were performed using Duncan's test or Dunnett's test. Fisher's exact test was used for comparisons of frequency data. Plasma P4 concentrations were greater (P<0.05) from Day 2.5 to Day 5.5 in the Day2-treated groups and from Day 3.5 to Day 5.5 in the Day3-treated cows than in the control group. CL area and blood flow from Day 2 to 8.5 did not differ (P>0.1) among groups, suggesting that there was no effect of P4 treatment on luteal development. The frequency of cows that began luteolysis before Day 15 was greater (P<0.04) in cows treated with 300 mg (4 out of 7 cows in each group) than in the controls (0 out of 7). The interval from pre-treatment ovulation to beginning of functional and structural luteolysis was shorter (P<0.01) in the combined P4-treated groups than in the control cows (14.3 ± 0.6 d vs. 17.9 ± 1.1 d, and 15.6 ± 0.6 d vs. 18.6 ± 0.7 d, respectively). Collectively, data indicate that an earlier exposure of the uterus to elevated P4 concentrations leads to anticipated onset of mechanisms involved in the luteolytic process. In conclusion, we propose that early P4 supplementation is not associated with a reduced CL size, vascularization or P4 secretion during luteal development, but anticipates on about 3 d the beginning of functional and structural luteolysis in beef cattle.

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A207 Embriology, Biology of Development and Physiology of Reproduction

### Effect of PDE5 inhibition on nuclear maturation of bovine oocytes *in vitro* cultured

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**Keywords:** maturation, phosphodiesterase, sildenafil.

Nuclear maturation comprises the reversal of the first meiosis block of the oocyte from germinal vesicle stage up to the second meiosis block in metaphase II (MII). It is well established that the decrease in cAMP levels is required for resumption of meiosis. Similarly, cGMP also mediates the resumption of meiosis in mammalian oocytes (Tornell *et al.*, 1990, *Acta Physiol. Scand.*, 139, 511-517). The levels of cGMP are balanced between synthesis by guanylate cyclase and degradation by cGMP-specific phosphodiesterases (PDEs; PDE5, PDE6 and PDE9; Kass *et al.*, 2007, *Cardiovasc. Res.*, 75, 303-314). The aim of this study was to evaluate the effect of inhibition of one of the cGMP-specific PDEs on the nuclear maturation of bovine oocytes *in vitro* to assess its participation on the process. PDE5 was chosen because a specific inhibitor is available and because we have detected its expression in bovine oocytes and *cumulus* cells (Schwarz K.L., 2011. PhD thesis, FZEA-USP, 106p). Therefore, a dose-response assay was performed with different concentrations of sildenafil (SIL; PDE5 inhibitor) added to the *in vitro* maturation (IVM) medium from the start of culture (0-22h) or from 11h of IVM culture (11-22h). *Cumulus*-oocyte complexes were aspirated from commercial abattoir ovaries and *in vitro* matured (groups of 20) in droplets (100  $\mu$ L) of TCM199 under mineral oil with different concentrations of SIL (0,  $10^{-7}$ ,  $10^{-5}$ ,  $10^{-3}$ M; Santa Cruz Biotecnology, Santa Cruz, USA). The control (C) consisted of oocytes matured without SIL. Culture was carried out for 22h at 38.5°C and 5% CO<sub>2</sub> in air. After IVM, the oocytes were denuded, stained (10  $\mu$ g/mL Hoescht 33342 for 15 min) and assessed under an epifluorescence microscope to determine the rate of nuclear maturation (MII%). Four replicates were performed and the results analyzed by linear regression (SAS v. 9.2), considering a significance level of 5%. In culture with SIL 0-22h, the MII% were 66.3, 74.1, 85.3 and 53.9% for 0 (n=80),  $10^{-7}$  (n=62),  $10^{-5}$  (n=51) and  $10^{-3}$ M (n=76) SIL, respectively. The  $10^{-7}$ M group was similar to C (P>0.05) and  $10^{-3}$ M reduced maturation (P<0.05). The  $10^{-5}$ M group, however, increased the maturation rate (P<0.05) compared to C. When SIL was added between 11-22h of IVM, MII% were 62.0, 45.4 and 60.7%, respectively, for  $10^{-7}$  (n=49),  $10^{-5}$  (n=63),  $10^{-3}$ M (n=79) SIL. The  $10^{-7}$  and  $10^{-3}$ M groups were similar to C (P>0.05), whereas  $10^{-5}$ M reduced MII% (P>0.05). In conclusion, a higher concentration of SIL inhibits maturation when added 0-22h, suggesting a greater effect on the initial period of maturation. On the other hand, the  $10^{-5}$ M SIL group showed stimulatory effect in 0-22h and inhibitory in 11-22h periods of culture, suggesting that SIL exerts different effects depending on the concentration and maturation phase evaluated. More studies are needed to clarify the role of cGMP and PDE5 in the control of meiosis in bovine oocytes.

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A208 Embriology, Biology of Development and Physiology of Reproduction

### Global gene expression in bovine oocytes submitted to heat shock during *in vitro* maturation

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**Keywords:** bovine, heat stress, oocyte.

Exposure of bovine oocytes to elevated temperature reduces oocyte maturation and developmental competence. The aim of this study was to evaluate global gene expression in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) oocytes exposed to elevated temperature during *in vitro* maturation (IVM). Nelore (NEL n = 13) and Holstein cows (HBW n = 14) were kept in a common installation under the same management conditions. Environmental parameters such as air temperature [ $28.05 \pm 0.28^\circ\text{C}$  (maximum) and  $16.88 \pm 0.29^\circ\text{C}$  (minimum)] and relative humidity [ $77.36 \pm 1.30\%$  (maximum) and  $44.71 \pm 1.29\%$  (minimum)] and physiological parameters such as rectal temperature ( $38.37 \pm 0.04^\circ\text{C}$  - NEL and  $38.23 \pm 0.05^\circ\text{C}$  - HBW) and respiratory rate ( $28.05 \pm 0.44$  bpm - NEL and  $29.99 \pm 0.49$  bpm - HBW) were monitored. Cows were submitted to follicular aspiration sessions and cumulus-oocyte complexes (COCs) recovered were submitted to control ( $38.5^\circ\text{C}$  for 22 hours) and heat shock ( $41^\circ\text{C}$  for 12 hours followed by  $38.5^\circ\text{C}$  for 10 hours) treatments during IVM (Roth and Hansen, 2005, Reproduction, 129, 235-244). Then, COCs were denuded and stored at  $-80^\circ\text{C}$  until genomic DNA microarray evaluation. Three pools of 25 oocytes were used per experimental group. Total RNA was extracted by RNeasy Mini Kit (Qiagen), samples were submitted to RNA amplification (MessageAmp II aRNA Amplification Kit, Ambion) to obtain 100 ng of mRNA and submitted microrarray assay (Affymetrix GeneChip Bovine Genome Array). Data were analyzed with the software *FlexArray* 1.6.1.1. and the *Affy* package developed in the language R. Genes with fold-change of at least 1.5 and  $P \leq 0.05$  were considered differentially expressed. Results indicated 68, 6 and 5 differentially expressed genes between the variables breed, temperature and interaction breed x temperature, respectively. According to the Ingenuity Pathways Analysis functional classification, genes differentially expressed regarding breed x temperature interaction were related to cell signaling (*OSMR*), lipid metabolism (*ACOXI*), protein processing (*CCT4*), gene expression control (*DICER1*) and protein catabolism (*DENND3*). Genes *CCT4*, *DICER1* and *ACOXI* were up-regulated in heat shocked HBW oocytes group (*CCT4*: HBW- $41^\circ\text{C}$  vs HBW- $38.5^\circ\text{C}$ ; *DICER1*: HBW- $41^\circ\text{C}$  vs NEL- $41^\circ\text{C}$  and *ACOXI*: HBW- $41^\circ\text{C}$  vs HBW- $38.5^\circ\text{C}$  and NEL- $41^\circ\text{C}$ ). *OSMR* gene was up-regulated in NEL- $41^\circ\text{C}$  oocytes as compared to HBW- $41^\circ\text{C}$  and the *DENND3* gene was up-regulated in NEL- $38.5^\circ\text{C}$  as compared to HBW- $38.5^\circ\text{C}$ . In conclusion, factors such as genotype and temperature modulate expression of genes that play key biological roles in cell growth, differentiation, protein and RNA processing. Functional studies will be needed to better characterize the thermoprotective role of these molecules.

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A209 Embriology, Biology of Development and Physiology of Reproduction

### Natriuretic peptides during ovulation in cattle

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**Keywords:** EGFR, LH, NPPC.

The Natriuretic Peptides (NPs) have been observed in the local regulation of reproductive functions of mammals, besides their systemic activity. Recently, the NPC precursor (NPPC) mRNA expression downregulation by hCG in granulosa cells has been demonstrated. Furthermore, NPPC mRNA expression is downregulated by amphiregulin in granulosa cells *in vitro*. In monovular species the role of NPs during ovulation is not fully understood. The aim of this study was to evaluate the pattern of NPs precursors (NPPs) and receptors (NPRs) mRNA expression in granulosa cells after GnRH-induced ovulation *in vivo* in cattle and their interaction with the EGF system *in vitro*. Cyclic beef cows were synchronized using a progesterone-based protocol. After intravaginal device removal (day 9), ovaries were examined by transrectal ultrasonography and cows that had GnRH-responsive preovulatory follicles ( $\geq 12$  mm) were challenged with 100  $\mu$ g gonadorelin acetate IM 12 h after removal of intravaginal device. Treated cows were then ovariectomized at 0, 3, 6, 12 and 24 h post-GnRH via colpotomy (n=5 to 6 animals in each time-point). Immediately after ovariectomy, follicular fluid was recovered and each cell type (granulosa and theca) was isolated. The effect of NPs on EGF-like factors (epiregulin and amphiregulin) mRNA expression and the effect of EGFR signaling blockade on LH modulation of NPPC mRNA expression was evaluated using granulosa cell culture. Data were tested for normal distribution using Shapiro-Wilk test and analyzed by ANOVA. NPPA mRNA expression was not regulated after GnRH treatment *in vivo* but its receptor (NPR1) expression increased ( $P < 0.05$ ) at 24 h compared to 0 h (time of GnRH treatment). The mRNA coding for NPPB was not detected in bovine granulosa cells. Interestingly, NPPC was increased at 3 and 6 h after GnRH treatment ( $P < 0.05$ ), returning to levels similar to hour 0 at 12 and 24 h whereas its receptor (NPR2) was not regulated. *In vitro*, granulosa cell treatment with NPA and NPC alone or combined with LH did not modulate amphiregulin and epiregulin expression. The addition of LH to granulosa cell culture induced NPPC mRNA expression ( $P < 0.05$ ), as observed *in vivo* after GnRH treatment, being LH effect completely abolished after addition of EGFR blocker (AG1478) to granulosa cell culture. In summary, NPPC mRNA is upregulated by LH *in vivo* and *in vitro* and the LH-effect on NPPC expression is mediated by activation of EGFR. These results suggest that NPs are involved in the ovulatory process in bovine and that the regulation and function of NPs during ovulation may differ between monovular and polyovular species.



A210 Embriology, Biology of Development and Physiology of Reproduction

### Occurrence of estrus in cycling bovine heifers after PGF2 $\alpha$ application

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**Keywords:** estrus, heifers, prostaglandin.

Prostaglandin F2 $\alpha$  (PG) and its synthetic analogues have been widely studied since its discovery in 1970, as potent luteolytic agents (Fierro et al., 2013, *Theriogenology*, 79, 399-408). The corpus luteum (CL) maturity at the time of PGF2 $\alpha$  application has a marked influence on the luteolytic response, however in the first six days of the estrous cycles there is no luteolytic effect of PGF2 $\alpha$  injection (Bó *et al.*, 2002, *Theriogenology*, 57, 53-72). In animals that respond to treatment with PG estrus is distributed in a period of six days (Macmillan K. L; Henderson H. V., 1984, *Anim Reprod Sci.*, 6, 245-254). Thus, the aim of this study was to evaluate the occurrence and time of estrus after PG application in cycling Nelore and crossbred heifers. We used 199 Nelore and 79 crossbred heifers. Heifers received i.m luteolytic dose of 0.526 mg sodium cloprostenol (Sincrocio<sup>®</sup>, Ourofino Saúde Animal, São Paulo, Brazil) using 3ml disposable syringe with 40x12 needle. The estrus detection started 24 hours after PG application in an observation schedule throughout one hour AM and one hour PM. The occurrence of estrus was 48.93% (n=136/ 278). Nevertheless, in Nelore heifers the occurrence of estrus was 45.23% (n=90/199) whereas in the crossbred heifers was 58.23% (n=46/79). Moreover the distribution of estrus after PG application occurred in 29 animals (27.55%) within 48 hours, 60 animals (43.13%) within 72 hours, 31 animals (22.45%) within 96 hours, 10 animals (7.32%) within 120 hours and in one animal (0.50%) within 144 hours. The estrus distribution among Nelore heifers occurred in 12 animals (6.03%) within 48 hours, 43 animals (21.1%) within 72 hours, 22 animals (11.06%) within 96 hours, 7 animals (3.52%) within 120 hours and one animal (0.50%) at 144 hours. Among crossbred heifers distribution of estrus occurred in 17 animals (21.52%) within 48 hours, 17 animals (21.52%) within 72 hours, 9 animals (11.39%) within 96 hours, 3 animals (3.80%) within 120 hours and none at 144 hours. The results show that cycling heifers respond to PG application and occurrence of estrus is distributed up to 144 hours, with the distribution especially from 48 to 96 after PG application.



A211 Embriology, Biology of Development and Physiology of Reproduction

### **Quantification of total lipids of *in vitro* produced bovine embryos with different developmental kinetics**

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**Keywords:** bovine, embryo, lipids.

The embryo morphology and cleavage and blastocyst rates have been used to assess embryo viability. However, with the advent of new biotechnologies, it has become clear that embryo viability can be severely compromised without noticeable morphological changes. One of these changes is the higher lipid accumulation in IVP embryos. This has been related with a lower cryopreservation efficiency and embryo viability, and could be an indicative of inadequate culture conditions when compared with the *in vivo* system. Based on these data, the hypothesis of this work was that embryos of different developmental kinetics have different characteristics of lipid accumulation, which is reflected in embryo viability. Our objective was to determine the amount of total lipids in embryos of fast (4 cells 40hpi and blastocysts from this group) and slow (2 cells 40hpi and blastocysts from this group) development by lipids staining at different stages of early development. For this, IVP fast and slow bovine embryos (individually cultured) and control (conventional group cultured) were used for total lipids staining by SUDAN BLACK B (Sigma) at cleavage (40hpi) and blastocyst stages (186hpi) (triplicate, n = 8 embryos per group minimum). The cleaved embryos and blastocysts were photographed and the images processed by threshold tool so to only lipids to become evident. After this transformation, we calculated the number of pixels obtained from each image and converted into arbitrary units by a script created in the development environment Matlab using the Image Processing toolbox. The results were submitted to ANOVA with Tukey post test (Prism 5 GraphPad Inc.). There was no difference between groups in the amounts of lipids among the cleavage groups (Fast:  $93\,884 \pm 4331$ ; Slow:  $68911 \pm 7180$ ; Fast Control:  $74622 \pm 21180$ ; Slow Control:  $70763 \pm 20046$ ). However, the amount of lipids was lower for slow blastocyst when compared with the control and fast groups (Slow:  $38617 \pm 3379$ ; Fast:  $122626 \pm 30378$ , Control:  $95658 \pm 15138$ ). There was no difference between the fast and control groups. These results show that the developmental kinetic and culture conditions have direct influence on lipid accumulation in IVP bovine embryos. Furthermore, these data can contribute to the improvement of the IVP system, especially for the production of embryos for cryopreservation.

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A212 Embriology, Biology of Development and Physiology of Reproduction

### Ovarian evaluation of *Trachemys scripta elegans* (WIED, 1839) turtles raised in Brazil

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**Keywords:** ovary, testudines, turtle.

*Trachemys scripta elegans* (*T. scripta elegans*) is an underwater turtle native to North America, however, in Brazil, it is invasive and exotic. The lack of knowledge on the reproductive biology of exotic species may interfere with population control measures or comparative studies. Therefore, this study aimed at describing the ovaries of *T. scripta elegans* turtles to contribute with information on their reproductive biology under Brazilian conditions. The macroscopic and topographic anatomy and the morphometry (mass (MO), volume (VO) and length (LO)) of the right (r) and left (l) ovaries of 40 turtles from the Parque Ecológico do Tietê<sup>1</sup> (IBAMA<sup>2</sup> Record Nr. 2491988) were studied. Mass (g) was estimated in analytical scale, volume (ml) according to the Scherle method (1970) and length using a millimetric precision caliper (Mitotoyo®). Ovarian follicles were determined macroscopically and classified in: Type 1 (T1 = 0.5 – 1.0 cm), Type 2 (T2 = > 1.0 to 2.5 cm) and Type 3 (T3 = > 2.5 cm). The Kruskal-Wallis test, followed by testing, was employed for comparison of means ± sd and simple correlation coefficient (R) was determined among variables (Assistat 7.6 beta). This study was approved by the CEDEP<sup>3</sup> from UNIVASF<sup>4</sup> (Protocol nr 0001/160412). The ovaries were irregular structures filled with developing follicles (primary, secondary and tertiary) on a richly vascularized stroma. They were located in the central region of the celomatic cavity, caudally to the intestine, stomach, liver and heart, medially to the uterine tubes, cranially to the urinary vesicle and cranioventrally to the swim bladder. MOd (11.95 ± 8.87 g), VOd (11.28 ± 8.72 ml) and LOd (12.52 ± 5.12 cm) were similar (P>0.05) to MOe (15.48 ± 9.89g), and to VOe (15.20 ± 9.37 ml) and to LOe (14.40 ± 4.61 cm). The average numbers of T1, T2 and T3 follicles were, respectively, 4.15±3.83, 3.80±3.61 and 0.60±1.26 on the right antimeres and 5.35±4.14, 4.25±4.29 and 0.70±1.76 on the left antimeres, and there was difference (P<0.01) among T1, T2 and T3 within the same ovary and among ovaries of different antimeres. High correlation (P<0.01) was observed among MO, VO, LO and T2 follicles within each ovary and among the T1 and T3 follicles among ovaries of different antimeres, while there was moderate correlation (P<0.05) between MO and VO and among T2 follicles in ovaries of different antimeres. It is concluded that in specimens studied, ovaries developed symmetrically, ovarian morphometry and the quantity of T2 follicles correlate highly and positively, ovaries had a higher amount of T1 follicles, followed by T2 and T3 and the left ovary showed higher quantity of each follicular type. This data provides scientific support on the reproductive biology of this exotic species, helping on its population control and comparative studies.

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A213 Embriology, Biology of Development and Physiology of Reproduction

### **ITS supplementation in maturation medium and low oxygen tension during *in vitro* culture improve blastocyst formation in domestic cat ICSI embryos but enhances DNA fragmentation**

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**Keywords:** DNA fragmentation, feline, ICSI.

The ICSI procedure is potentially of great value for felids, and it has not been extensively studied in these species. The objective of this work was to evaluate embryo development and DNA fragmentation of cat ICSI embryos treated with antioxidant conditions during oocyte maturation and embryo culture. Ovaries were recovered from cats subjected to ovariectomy. Cumulus-oocyte-complexes were *in vitro* matured in maturation medium (MM): TCM199 containing 1 IU/ml HCG, 10 ng/ml ECG, 2.2 mM calcium lactate, 0.3 mM pyruvate, 0.3% BSA and 3% antibiotic-antimycotic; or MM supplemented with 1 $\mu$ l/ml of the insulin, transferrin and selenium (ITS, a free radical reducer). After ICSI, presumptive zygotes were cultured in SOF at 39°C and atmospheric oxygen tension (21%O<sub>2</sub>) or low oxygen tension (5% O<sub>2</sub>). The experimental groups were: MM-21%O<sub>2</sub> (n=138), MM-5%O<sub>2</sub> (n=142), ITS-21%O<sub>2</sub> (154) and ITS-5%O<sub>2</sub> (n=206). Control SHAM groups were included for each treatment. We evaluated cleavage on day 2 and blastocyst formation on day 7. The blastocysts from all the ICSI groups were evaluated by TUNEL assay to determine total cell number and the presence of fragmented nuclei. *In vitro* embryo development was compared by non-parametric Fisher's exact test, differences in total cell number by one-way ANOVA and the proportion of fragmented nuclei over total cell number by the "difference of proportions test", (p<0.05). The cleavage rates were lower (p<0.05) in the ITS-21%O<sub>2</sub> group (35.7%) than the other three ICSI groups (52.2%, 55.6% and 56.8% for MM-21%O<sub>2</sub>, MM-5%O<sub>2</sub> and ITS-5%O<sub>2</sub>, respectively). Regarding blastocyst formation, the highest blastocyst rate was observed in the group ITS-5%O<sub>2</sub> (20.9%) vs. 8.7%, 7% and 6.5% for MM-21%O<sub>2</sub>, MM-5%O<sub>2</sub> and ITS-21%O<sub>2</sub>, respectively. No blastocyst development was observed in any of the SHAM groups. The mean of blastocyst cell number did not differ among the groups (177.8±28.7, 105.9±16.7, 128.6±18.3 and 129.4±17.9 for MM-21%O<sub>2</sub>, MM-5%O<sub>2</sub>, ITS-21%O<sub>2</sub> and ITS-5%O<sub>2</sub>, respectively). However, the proportion of TUNEL+ cells was statistically higher in the group ITS-5%O<sub>2</sub> (67.6%), in respect to the other three groups (43.5%, 36.5% and 34% for MM-21%O<sub>2</sub>, MM-5%O<sub>2</sub> and ITS-21%O<sub>2</sub>, respectively). Our results showed that the antioxidant conditions used in this study (ITS and low oxygen tension) improved embryo development *in vitro* but increased DNA fragmentation. It is possible that embryo development improved because of higher sperm decondensation in oocytes matured with ITS, as has been previously reported (Yeon et al. 2006, Animal Reproduction Science, 106:13-24), and more physiological conditions using low oxygen tension during culture. We also suggest that these conditions allowed the blastocyst formation of embryos of lower quality and higher DNA fragmentation that would not have developed in the other conditions. More studies are needed to determine the best conditions to generate viable cats after the transfer of ICSI embryos to recipient females.



A214 Embriology, Biology of Development and Physiology of Reproduction

### **Pharmacological blockade of H3K27 trimethylation increases cell apoptosis of in vitro-produced porcine embryos**

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**Keywords:** differentiation, epigenetics, polycomb.

Trimethylation of lysine 27 of histone H3 (H3K27me3) is an epigenetic mark that mediates transcriptional repression and controls pluripotency in embryonic cells. This effect occurs through the temporary repression of genes that control embryo development and differentiation of pluripotent cells. Trimethylation of H3K27 is catalyzed by proteins of the Polycomb Repressive Complex 2 (PRC2), however, this process is not entirely known. 3-Deazaneplanocin A (DZNep) can inhibit the action of PRC2, thus preventing H3K27 trimethylation. The aim of this study was to investigate the effect of adding DZNep to the in vitro culture media of porcine embryos. Oocytes derived from ovaries collected in an abattoir were fertilized in vitro and cultured with 0 (control group; n = 101) or 5  $\mu$ M DZNep, starting on D2 (n = 108) or D4 (n = 120) of embryo culture. Embryos were fixed on D8 and submitted to an immunofluorescence protocol, with rabbit anti-cleaved caspase-3 monoclonal primary antibody and Alexa Flour 488 goat anti-rabbit secondary antibody. The cellular DNA was stained with DAPI. The total cell number and the number of apoptotic cells were determined and the rates of apoptosis between the different treatments were compared using the Chi-square test, with 5% as the significance level. The rate of apoptosis in the control group was 21.4%, lower than the ones of DZNep D2 (45.5%) and DZNep D4 (31.4%) groups. Apoptosis in DZNep D2 group was higher than in DZNep D4 ( $P < 0.05$ ). These data show that, when the inhibitor of PRC2 is added at the beginning of the embryo culture, the induction of apoptosis is more significant and embryonic development is affected more severely. Based on these results, it can be concluded that the Polycomb group proteins play an important role in the regulation of early development of porcine embryos, since their inhibition results in increased apoptosis rate of the embryonic cells. It can also be inferred that, the earlier in embryonic development is the blocking of this complex, the greater is the rate of cellular apoptosis induced.



A215 Embriology, Biology of Development and Physiology of Reproduction

### Effect of melatonin on *in vitro* maturation of bovine oocytes

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**Keywords:** *in vitro* maturation, melatonin, recombinant FSH.

*In vitro* maturation (IVM) is a technique that allows the rescue of large amounts of immature oocytes from the ovary and the culture to produce *in vitro* developed embryos. However, not all oocytes removed from the ovarian follicles acquire full nuclear and cytoplasmic competence. Melatonin is a hormone that exhibits antioxidant and antiapoptotic properties, acting in cell protection (Hardeland R. et al., 1993, *Neurosci Biobehavioral Rev.*, 17, 347–357), besides regulating several signaling pathways (Tamura et al, 2009, *Fert Steril*, 92, 328-343). Thus, the addition of melatonin during IVM could be used to improve the competence of bovine oocytes. The objective of this study was to investigate the influence of melatonin on the progression of nuclear maturation *in vitro*. *Cumulus*-oocyte complexes (COCs) were aspirated from slaughterhouse ovaries, selected and transferred in groups of 25 to 100  $\mu$ L droplets of IVM medium [TCM-199 with 0.1% polyvinyl alcohol (PVA), sodium pyruvate (25 mM) and gentamicin (25  $\mu$ g/ml)] under mineral oil. For the experiments, the maturation medium was supplemented with recombinant FSH (0.5  $\mu$ g/ml, positive control) or melatonin ( $10^{-6}$  and  $10^{-9}$ M) and cultured for 6, 12, 18 and 24 h at 38.5°C and 5% CO<sub>2</sub> in air. As an additional control, a group was matured without addition of FSH or melatonin. For the analysis of germinal vesicle breakdown rate (GVBD), the oocytes cultured for 6 and 12 h were labeled with anti-lamin A/C-DAPI (Prentice-Biensch et al., 2012, *Theriogenology*, 78, 1633–1638) and to analyze the rate of maturation (metaphase II, MII) oocytes cultured for 18 h and 24 h were stained with Hoechst 33342 (10  $\mu$ g/ml, 15 min). Data from three replicates were analyzed by Chi-square at a significance level of 5%. As expected, all oocytes in groups matured for 6 h remained immature in germinal vesicle (GV). For groups cultured for 12 h, the control showed a lower proportion of oocytes in GVBD (n=50; 46%, P <0.05) than the other groups (FSH or melatonin), which were similar among themselves (n=57 to 68; 66.1 to 78.9%, p>0.05). At 18 h of IVM, FSH group had MII rates (n=57; 73.6, P<0.05) superior to  $10^{-9}$ M (n=42; 52.3%), while the others were similar to both (n=41 and 47; 71 and 57.4%, respectively for the control and  $10^{-6}$ M, P>0.05). At 24 h,  $10^{-9}$ M (n=45; 46.6%, P<0.05) was inferior to the others (n=44 to 58; 70.4 to 86.2%, P>0.05). In conclusion, meiosis resumption was stimulated by melatonin in a similar manner as the addition of FSH. However, meiosis progression was stimulated only by the highest concentration of melatonin. Therefore, the results suggest a possible role of melatonin in the control of oocyte maturation in cattle. Further studies are in development to evaluate the role of melatonin in cytoplasmic maturation and its antiapoptotic and antioxidant functions in bovine oocytes.

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A216 Embriology, Biology of Development and Physiology of Reproduction

## **Sperm pretreatment prior to ICSI is not necessary for an adequate *in vitro* bovine embryo development**

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**Keywords:** bovine, embryo, ICSI.

Efficiency of ICSI in bovine is lower than in other species due in part to a lack of optimal conditions for its implementation, which has prevented to achieve high rates of embryonic development and the birth of live offspring. The aim of this study was to evaluate the effects of pretreatments of bovine sperm on the *in vitro* developmental potential of embryos generated by ICSI. Cumulus-oocyte complexes were aspirated from follicles 2-8 mm in diameter and *in vitro* matured. Motile sperm were selected by Percoll gradient technique. Sperm were treated with 1 mM NaOH for 1h or 5 mM DTT 20 min and sp-TALP for 120 min at 38.5°C, respectively. ICSI was performed in microdrops under mineral oil using a Nikon TS100 inverted microscope. Each sperm was selected and subjected to tail scoring before being aspirated into the injection pipette (9 mm internal diameter). The injected oocytes were activated by exposure to 10 µM ionomycin for 5 min and 5 µg/ml cycloheximide for 5 h and cultured in drops of KSOM medium (culture medium regularly used in our laboratory) under mineral oil at 38.5°C and 5% CO<sub>2</sub>. Cleavage was recorded at 72 h, blastocysts rate on day 9 and pronuclear formation was evaluated at 18 h post activation (Hoescht staining; Bevacqua et al., 2010, *Theriogenology*, 74, 922-31). Quality of embryos was assessed by staining with Hoechst and propidium iodide (Fouladi-Nashta et al., 2005, *Reproductive BioMedicine Online*, 10, 497-502). The proportional data were transformed to arcsine, treatment effects were analyzed by ANOVA and means were compared using Scheffe test. Pronuclear formation was analyzed by a Chi-square test with Bonferroni's correction. Results of 8 replicates with a total of 455 oocytes injected with spermatozoa pretreated with DTT (ICSI-DTT), NaOH (ICSI-NaOH) and sp-TALP (ICSI-ST) showed no differences in the cleavage rate in any of the groups (69, 71 and 69%, for ICSI-ST, ICSI-DTT and ICSI-NaOH, respectively). Similar results were observed in the blastocysts rate (29, 22 and 20% de blastocistos for ICSI-ST, ICSI-DTT and ICSI-NaOH, respectively). The fertilization rate observed, as assessed by the presence of male (or decondensed sperm head) and female pronucleus was 77, 77 and 67% for ICS-ST, ICSI-DTT and ICSI-NaOH, respectively. Quality of embryos was not different between treatments. In conclusion, we describe here for the first time the effects of NaOH treatment on *in vitro* embryonic development after ICSI and demonstrated that classical sperm pretreatment with DTT is not essential for an appropriate *in vitro* embryo development in the bovine species.

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A217 Embriology, Biology of Development and Physiology of Reproduction

### Natriuretic peptides stimulate the cumulus oophorus expansion in bovine

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**Keywords:** cumulus expansion, forskolin, natriuretic peptides.

The cumulus-oocyte complex (COC) is influenced by the preovulatory peak of LH, which in addition to initiating meiosis resumption in the oocyte, induces expansion of the compact layer of *cumulus* cells that surrounding it. However, the absence of this gonadotropin receptors in COCs (Peng et al., 1991, Endocrinology, 129, 3200-3207) suggests that LH does not act directly on the female gamete, but by stimulating intrafollicular mediators that act on a paracrine way (Park *et al.*, 2004, Science, 303, 682-684). The Natriuretic Peptides (NP) were discovered after infusion, in rats, of atrial tissue extract, causing rapid decrease in blood pressure, vasodilation and increased urinary sodium excretion (De Bold et al., 1981, Life Science, 28, 89-94). In mice, it is known that the C-type NP (NPC) has the capacity to block meiosis resumption (Zhang et al. 2010, Science, 330, 366-369), but there is no effect proven on *cumulus* expansion. Similarly, there is little knowledge of forskolin and NP on this function. The objective of this research is to propose an *in vitro* model for the study of bovine *cumulus* expansion and evaluate the effect of NP on this process. The COCs were aspirated from abattoir ovaries, using grade 1 and 2 oocytes. Each COC had images captured and their total area ( $\mu\text{m}^2/\text{CCO}$ , LeicaApplication) measured immediately before (0h) and at the end of the culture period (10 COCs per well). Data were evaluated using randomized blocks, with different treatments performed simultaneously and each replication considered as a block, and the results were processed by PROC RANK for the purpose of applying parametric tests. Experiment 1: groups of COCs were allocated to 12 or 24h of culture with TCM alone; TCM+0.5 $\mu\text{g}/\text{ml}$  FSH; TCM+100 $\mu\text{M}$  forskolin; TCM+100 $\mu\text{M}$  forskolin+0.5 $\mu\text{g}/\text{ml}$  FSH. Experiment 2: we evaluated the dose-response effect (10, 100 or 1000nM) of each NP, for 12h of maturation in stimulating *cumulus* expansion inhibited by 100 $\mu\text{M}$  forskolin. In all experiments the oocytes started culture with similar sizes. In experiment 1, after 12h of *in vitro* maturation the group TCM+0.5 $\mu\text{g}/\text{ml}$  FSH showed the highest growth ( $P < 0.05$ ). At that time, the TCM, TCM+100 $\mu\text{M}$  forskolin+0.5 $\mu\text{g}/\text{ml}$  FSH and TCM+100 $\mu\text{M}$  forskolin were not different ( $P > 0.05$ ). After 24h of culture the effect observed in group TCM+100 $\mu\text{M}$  forskolin was not maintained. In experiment 2, after 12h of *in vitro* maturation the positive control group (TCM+0.5 $\mu\text{g}/\text{ml}$  de FSH), 1000nm ANP, 100nm BNP and 10nm CNP showed total area similar and higher than the negative control (TCM+100 $\mu\text{M}$  de Forskolin+0.5 $\mu\text{g}/\text{ml}$  de FSH). These results validate a model for *in vitro* study of *cumulus* cells expansion in bovine using forskolin, and furthermore, demonstrated the involvement of ANP, BNP and CNP in *cumulus* expansion.



A218 Embriology, Biology of Development and Physiology of Reproduction

## Ooplasmic tranfer on the development of zona-free IVF bovine embryos

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**Keywords:** Bovine, IVF, ooplasmic transfer.

Ooplasmic transfer (OT) has been mainly used to improve compromised oocytes and SCNT embryos development. The objective of this work was to evaluate the effect of the OT on development and quality of zona-free IVF embryos (ZF-IVF) and their individual blastomeres (IB) after disaggregation on day 1 post-insemination. COCs were aspirated from slaughterhouses ovaries and selected for standard IVM. A group of oocytes was subjected to IVF and presumptive zygotes were denuded and ZP removed. During gametes coincubation, a second group of matured oocytes was subjected to denudation and to ZP removal prior to enucleation. One (1+ group) or two (2+ group) ooplasms were fused to one presumptive zygote (2 pulses of 60V during 30 usec length and 100 msec interval). A ZF-IVF group (without ooplasm fusion) and a standard IVF (ZP-IVF) group were used as controls. For evaluation of IB development, cleaved embryos at day 1 post-insemination from ZF-IVF, 1+ and 2+ were subjected to hard pipetting for blastomeres disaggregation. ZF-zygotes and IB were cultured using the WOW system. Cleavage and cell numbers of ZF embryos were evaluated on Day 2. Blastocysts rates and total cell numbers were evaluated on Day 7 in all groups. Data were analyzed by Fisher's test ( $p < 0.05$ ). The groups 1+ and 2+ showed a higher number of cells ( $>9$  cells) on Day 2 (62/144; 43% and 49/138; 35.5%) than the ZF-IVF embryos (81/318; 25.5%,  $p < 0.05$ ). On the contrary, a higher proportion of cleaved ZF-IVF embryos showed 5 to 8 cells on Day 2 (149/318; 47%). The overall cleavage and blastocyst rates were significantly higher in the ZF-IVF (88% and 24%) and the 1+ (82% and 25%) groups than in the 2+ group (61% and 14%). The ZP-IVF group showed the highest blastocysts rates (131/343; 38%). Surprisingly, 1+ and 2+ groups showed blastocyst cell numbers ( $60.8 \pm 16.38$  and  $56.50 \pm 26.00$ , respectively) similar to the ZP-IVF group ( $58.26 \pm 6.65$ ) and higher ( $p < 0.05$ ) than observed in the ZF-IVF control blastocysts ( $43.94 \pm 11.69$ ). Interestingly, the highest percentage of blastocysts was obtained in groups showing an increased proportion of cells on Day 2: 62% of the blastocysts in 1+ group were obtained from embryos that were in 9 to 16-cell-stage, while 50% of the blastocysts of 2+ group had more than 16 cells-stage on Day 2. A total of 20 disaggregated embryos (49 IB) in the ZF-IVF group resulted in 24 blastocysts (120%); significantly higher than the other experimental groups (27% and 0% for the 1+ and 2+ group, respectively). Additional experiments are being carried out to identify the effects of OT in zygotes in terms of transcriptional pattern, pregnancy establishment and post-vitrification survival. In addition, we confirmed in all groups a positive correlation between more advanced stages of development at Day 2 and higher blastocysts rates. However, 1+ and 2+ reconstructed embryos did not improve blastomeres development. In conclusion, OT improved embryo development when 1 ooplasm (1+) was added, but 2 ooplasms transfer (2+) showed to be excessive and harmful to the embryo.



A219 Embriology, Biology of Development and Physiology of Reproduction

### **The natriuretic peptides system is present in the cumulus-oocyte complex and stimulates the meiosis resumption in bovine**

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**Keywords:** forskolin, Natriuretic peptides, oocyte maturation.

The process of meiosis resumption in oocytes, blocked since fetal life, is triggered by the preovulatory peak of LH. However, the absence of this gonadotropin receptors in cumulus-oocyte complexes (COCs; Peng et al., 1991, *Endocrinology*, 129, 3200-3207) suggests that LH does not act directly on the female gamete, but stimulating intrafollicular mediators that act of paracrine way (Park *et al.*, 2004, *Science*, 303, 682-684). Among the components of natriuretic peptides (NP) system, only the NP C-type (NPC) has demonstrated its capability to block meiosis resumption in mice (Zhang *et al.*, 2010, *Science*, 330, 366-369). However, there is no knowledge about the role of this system in controlling meiosis in monovular species. The aim of this study was to characterize the system natriuretic peptides in COCs and demonstrate its role in meiosis resumption in bovine oocyte. The COCs were aspirated from abattoir ovaries, selected (grade 1 and 2) and immediately used for the characterization of the NP system by PCR (experiment 1) or cultured for 12h in TCM199. In experiment 2, the maturation medium was supplemented with different doses (10, 100 and 1000nm) of NP A-type (ANP), B-type (BNP) and C-type (CNP) to evaluate the effect on blocking the meiosis resumption. In the third experiment, the oocytes remained arrested in germinal vesicle (GV) by action of forskolin (100µM; for all maturation), and it was evaluated the dose-response effect (10, 100 and 1000nm) and possible associations of the three NP in stimulating the meiosis resumption. After the maturation period (12h) the oocyte meiotic progression was visualized using Hoechst 33342. The differences between treatments were calculated using a statistical model for categorical data (PROC CATMOD). Initially, we demonstrated the presence of mRNA for ANP, CNP, and the natriuretic peptide receptor 1 (NPR-1), NPR-2 and NPR-3 in cumulus cells, and only NPR-2 mRNA in oocyte. In the second experiment, any dose or association of NP were able to maintain meiotic arrest. In experiment 3, ANP (1000nm), BNP (10 nM) and CNP (1000nm), induced meiotic resumption (73.4, 66.6 and 58.8%, respectively) after 12h of maturation compared to the negative control (24,0%), and moreover, we observed that association ANP + BNP (62.9%), ANP + CNP (69.6%), BNP, CNP + (62.5%) and ANP + BNP + CNP (64 9%) were effective in stimulating the meiosis resumption, but with similar rates seen when used each NP separately. Therefore, we demonstrate for the first time the NP system in COCs, and functional studies suggest that in monovular species such as bovine, the NPs are involved in stimulating the meiosis resumption.



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### Effect of preovulatory follicle size on the pathway of eicosanoids biosynthesis in the endometrium of Nelore cows during early diestrus

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**Keywords:** cattle, estradiol, progesterone.

In cows, different estradiol and progesterone levels during the periovulatory period act distinctly on uterine tissues and modulate its function through the control of several metabolic pathways. For example, there is evidence that these steroids modulate the synthesis and signaling of eicosanoids. Such compounds are derived from the arachidonic acid and are essential for a range of reproductive processes. It is believed that distinct eicosanoids profiles during the pre-implantation period modulate uterine receptivity and consequent pregnancy success. The objectives were to (1) measure the gene expression of proteins involved in the synthesis, transport and signaling of eicosanoids and (2) quantify a series of eicosanoids in the uterus under different periovulatory hormonal profiles during early diestrus in beef cows. The follicular growth of multiparous non-lactating Nelore cows was pharmacologically manipulated in order to obtain groups with large (LF/CL; n=11) or small (SF/CL; n=11) preovulatory follicles and corpora luteum (Mesquita F. *Reprod. Fertil. Dev.*, submitted). Uterine wash and tissues were obtained seven days after the induction of ovulation with GnRH analogue. Abundance of transcripts from 28 genes involved in eicosanoids synthesis was measured by qPCR and the concentration of metabolites in the endometrial tissue (in pmol/g of tissue) and uterine wash (in pmol/mL of wash) by mass spectrometry. In the LF/CL group, there was a greater abundance of transcripts coded by the genes ALOX12 (1.56±0.09 vs 1.07±0.10; p<0.05), PTGIS (1.22±0.09 vs 1.04±0.08; p<0.05), PTGES (1.15±0.10 vs 0.90±0.08; p<0.05), PTGES2 (1.18±0.08 vs 1.03±0.05; p=0.07), AKR1C4 (2.07±0.30 vs 1.12±0.15; p<0.05), and CBR1 (1.14±0.13 vs 0.83±0.12; p=0.07), responsible for the synthesis of eicosanoids. Identification and quantification of 65 arachidonic acid metabolites was performed in the endometrium and 87 in the uterine wash. However, the quantification of the respective eicosanoids in the wash did not differ (p>0.05) between the LF/CL and SF/CL groups in the compounds 12 HETE (0.6±0.1 vs 0.4±0.1), 6-keto-PGF1 $\alpha$  (142.9±2.7 vs 203.7±62.7), 8-iso-PGE2 (3.9±0.9 vs 3.3±1.3), PGE2 (27±6.4 vs 27±4.0) and PGF2 $\alpha$  (41.5±4 vs 49.7±6.8) in the endometrium and neither the compounds 12 HETE (1.3±0.6 vs 1.7±1.0), 6-keto-PGF1 $\alpha$  (1.4±0.4 vs 1.4±0.5) 8-iso-PGE2 (0.02±0.0 vs 0.01±0.0), PGE2 (0.3±0.1 vs 0.2±0.1) and PGF2 $\alpha$  (1.9±0.6 vs 1.8±0.8). Despite the LF/CL group expressed greater amounts of transcripts for the synthases enzymes than the SF/CL group, such differences did not reflect in the corresponding eicosanoids composition in the uterus. Therefore, the exposure of uterus to different fluctuations of ovarian steroids modulates the gene expression of enzymes linked to the eicosanoid synthesis without influencing the concentration of such compounds in the uterine wash and tissues in the beginning of estrous cycle in beef cows.

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**In vitro culture of bovine embryos in medium without FBS and supplemented with fatty acids and antioxidants: implications on the development, accumulation of lipids, total cell number and percentage of apoptotic cells**

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**Keywords:** antioxidant, fatty acids, IVC.

Different systems of culture and especially the medium composition for *in vitro* culture (IVC) in embryos are the main causes of great variation in the production rates and embryo quality (Thompson & Peterson, 2000). The increased lipid content in embryos caused by the use of FBS causes morphological changes and alterations in gene expression (Lonergan et al., 2003). It is proven that the use of conjugated linoleic acid (CLA) causes a reduction in lipid content (Pereira et al., 2008). Catalase (CAT) is an antioxidant and is responsible for extracellular removal of hydrogen peroxide from the culture medium (Fridovich, 1998). This study was conducted to evaluate the effect of supplementation with conjugated linoleic acid (CLA) and/or catalase in the culture medium in the absence of FBS. COCs (n=1.094) were matured in B199 (TCM-199 with bicarbonate and hormones) supplemented with 10% FBS. After IVF, zygotes were IVC in SOFaa with 8mg/ml BSA-FAF, 100  $\mu$ M CLA and/or 100 UI catalase in atmosphere of 5% CO<sub>2</sub> in air. Cleavage was evaluated at 48hpi and blastocysts rates at 168hpi when they were stained with Nile Red (SIGMA) for determination of the lipid content and TUNEL “*In situ terminal deoxynucleotidyl transferase mediated dUTP Nick and labeling assay*”, Roche Applied, IN, USA for apoptosis determination. Embryos were evaluated under an epifluorescent microscope and images of embryos stained with Nile Red were analyzed by Q-Pro Image Capture software for determination of the fluorescence intensity. Data were analyzed by ANOVA (P<0.05) and the averages compared by Tukey-Kramer HSD. The results are presented as mean  $\pm$  standard error of the mean. The cleavage rates were 82.5 $\pm$ 2.7%<sup>a</sup> (BSA), 84.2 $\pm$ 0.5%<sup>a</sup> (BSA+CLA), 86.2 $\pm$ 1.8%<sup>a</sup> (BSA+CAT) and 83.9 $\pm$ 2.5%<sup>a</sup> (BSA+CLA+CAT). Blastocysts rates were 18.7 $\pm$ 4.7%<sup>a</sup>, 14.9 $\pm$ 2.6%<sup>a</sup>, 8.3 $\pm$ 1.6%<sup>a</sup>, 6.4 $\pm$ 0.6%<sup>a</sup>, respectively. The fluorescence intensities with Nile Red were similar (P>0.05) among groups (0.99 $\pm$ 0.08 to 1.04 $\pm$ 0.15), as well as the percentage of apoptotic cells (0.36 $\pm$ 0.26 to 1.72 $\pm$ 0.49). The average number of total cells differed, being 60,0<sup>b</sup> (BSA), 110,4<sup>a</sup> (BSA+CLA), 66,0<sup>ab</sup> (BSA+CAT), 95,7<sup>a</sup> (BSA+CLA+CAT). Although the rate of embryo production did not differ between groups, the average results were lower than expected. This confirms that the presence of FBS is still essential to promote embryonic development, even if it is added in small amounts. The addition of CLA and CAT during IVC neither altered the amount of lipids of embryos, nor affected the percentage of apoptotic cells. In conclusion, supplementation of CAT and/or CLA in IVC medium without addition of FBS did not improve the rate of blastocyst development or embryo quality. However, supplementation with CLA or CLA+CAT reflected in an increase in the number of blastomeres.



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### **Effect of dietary supplementation with polyunsaturated fatty acids on the recovery and oocyte quality and serum concentration of progesterone, insulin and leptin**

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**Keywords:** bovine, hormones, polyunsaturated fatty acids.

The nutritional status can influence reproduction, especially fertility in bovine females. In dairy and beef cattle, dietary supplementation with various fat sources has been performed to increase the energy density of the diet and, consequently, improve reproductive performance, especially oocyte quality and by promoting the secretion of progesterone and other hormones. The aim of this study was to evaluate the effects of dietary supplementation of Nelore heifers with a source of rumen protected fat enriched with Omega-6 (n-6) and 3 (n-3) PUFAs (Megalac-E ®) on the follicular population, oocyte quality, and plasma concentrations of progesterone (P<sub>4</sub>), insulin and leptin. Sixteen heifers were randomly divided in two groups in a "cross-over" experiment, in which isoenergetic and isoproteic diets were used as follows: CONTR (n = 8, maintenance diet) and FAT (n = 8, diet for maintenance + 100 g / animal / day Megalac-E ® in its composition). After 60 days of feeding, ultrasound-guided ovum pick-up (OPU) sessions were carried out. A total of six sessions of OPU was performed during the experiment. The follicular population was assessed (counting and measurement of follicular diameter) with the aid of an ultrasound machine Pie Medical ® model Falcon 100. All follicles larger than 4 mm were aspirated to recover cumulus-oocyte complexes that were classified according to the manual of IETS in grades I, II, III, denuded and degenerated. Blood samples were taken at the beginning and after 60 days of dietary supplementation, as well as during OPU sessions. The hormone quantification was determined by radioimmunoassay using previously validated commercial kits. The mean (least squares ± SEM) were analyzed by the GLIMMIX procedure of SAS. There was no difference (P > 0.05) in the mean concentration of P<sub>4</sub> (2.6 ± 0.6 vs 3.5 ± 0.6 ng/mL), insulin (13.8 ± 1.6 vs 14.9 ± 1.6 ng/ml), and leptin (2.5 ± 0.3 vs 2.3 ± 0.3 ng/mL), respectively, for CONTR and FAT groups. Likewise, there was no difference in the population of follicles aspirated (17.9 ± 1.0 vs 15.8 ± 1.0), number of oocytes recovered (14.4 ± 1.4 vs 14.5 ± 1.3), and number of viable oocytes (12.1 ± 1.2 vs 12.5 ± 1.1), at the OPU sessions for CONTR and FAT groups, respectively. In conclusion, supplementation with a source of rumen protected fat rich in PUFAs in Nelore heifers had no effect on the recovery and quality of oocytes, as well as on the plasma concentrations of progesterone, insulin and leptin evaluated in this study.



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### **Methylation pattern of the XIST gene in oocytes from Nelore cows (*Bos taurus indicus*) during oogenesis**

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**Keywords:** DNA methylation, oogenesis, XIST.

DNA methylation is one of the most studied epigenetic events (Zaid et al., 2010, *Molecular and Cellular Biology*, 30, 4758-66; Guseva et al., 2012, *Developmental Biology*, 361, 403-11) being responsible for the epigenetic reprogramming that occurs during gametogenesis (Faulk et al., 2011, *Epigenetics*, 6, 791-7). Understanding how this reprogramming occurs in oogenesis is important to comprehend physiological and genetic aspects involved in female gametogenesis in order to create parameters for oocyte competence. This is important to improve the *in vitro* embryo production, maximizing the use of gametes. The aim of this study was to evaluate the DNA methylation pattern in Differentially Methylated Regions (DMR) involved in the control of XIST gene expression in oocytes from preantral and antral follicles of Nelore cows. The extracted DNA from oocytes was treated with sodium bisulphite and amplified by PCR for the XIST gene, which was cloned into DH5 $\alpha$  cells, and then purified and sequenced. The sequences were compared with that sequence in the *GenBank*, and only sequences with a minimum of 90% of homology and 90% of sodium bisulphite conversion were used. The statistical analysis was done using *Kruskal-Wallis* test, followed by the *Mann-Whitney* test. The methylation patterns found for oocytes of primordial, secondary, incompetent antral and competent antral follicles were  $91.59 \pm 6.4\%$ ,  $85.70 \pm 19.6\%$ ,  $91.25 \pm 7.2\%$  and  $92.58 \pm 11.7\%$ , respectively, for XIST gene. The hypermethylated pattern of XIST gene suggests that this event may be responsible for epigenetic reactivation of the X chromosome during oogenesis, which is observed in mouse MII oocytes (Kim *et al.*, 2009, *Nucleic Acids Research*, 37, 5656-64) and human MII oocytes (Nesterova et al., 2002 *Differentiation, research in biological diversity*, 69, 216-25). This suggests that the analyzed region is not undergoing an epigenetic reprogramming process during oogenesis.



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### **Intracellular signaling of angiotensin in the control of bovine oocyte nuclear maturation**

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**Keywords:** angiotensin, maturation, oocyte.

Mammalian target of rapamycin (mTOR) is an important intracellular pathway in the control of cell growth and proliferation by growth factors and nutrients stimuli (Murakami et al., 2004, *Molecular and Cellular Biology*, 24, 6710-6718; Laplante et al., 2012, *Cell*, 149, 274-293). The inhibition of this intracellular signaling by rapamycin negatively affects oocyte nuclear maturation in mice (Lee et al., 2012, *Molecular Reproduction and Development*, 79, 356-366). In bovine, the same factors and intracellular pathways have been studied during oocyte nuclear maturation process. During culture of cumulus oocyte complex (COCs) with follicular hemisections, angiotensin II (AngII) reverts the inhibitory effect of follicular cells on oocyte nuclear maturation (Giometti et al., 2005, *Theriogenology*, 63, 1014-25). The aim of this study was to assess the effects of mTOR intracellular pathway on AngII signaling during bovine oocyte nuclear maturation. Cumulus oocyte-complexes were aspirated from local abattoir ovaries. To obtain follicle hemisections, follicles with 2 to 5mm in diameter were isolated from the ovaries and dissected free of stromal tissue as previously described (Richard e Sirard, 1996, *Biology of Reproduction*, 54, 22-28). In the first experiment, COCs were cultured during 15 hours in 200µl TCM199 supplemented with rapamycin in different concentrations (2, 10 e 50µM) and further analysis of meiosis progression. In the second experiment, COCs were cultured as the following treatments: without follicular hemisections in the culture (positive control), or in the presence of follicular hemisections (negative control), plus AngII ( $10^{-9}$ ) and plus AngII ( $10^{-9}$ M) + saralasin ( $10^{-5}$ M). In the other groups rapamycin (2, 10 and 50µM) plus AngII ( $10^{-9}$ M) were used in the culture medium supplemented with follicular hemisections. After culture, the cumulus cells were removed by vortexing and oocytes were stained with Hoescht 33342 to assess meiosis progression. The percentage of meiosis progression were tested by ANOVA (PROC GLM). In the first experiment the number of oocytes that reached MI were significantly lower ( $P<0.001$ ) in the rapamycin (50µM) treatment group (28.57%) when compared to the control (90,19%). With follicular hemisections in the culture, the rapamycin at 10 and 50µM inhibited the AngII effect to promote meiosis progression (36.53 and 31.57%), respectively, likewise the group AngII ( $10^{-9}$ M) + saralasin( $10^{-5}$ M) (36.36%), but with significant difference when compared to hemisections plus AngII ( $10^{-9}$ ) group (56.6%;  $P<0.05$ ). These data contribute to obtain knowledge on AngII intracellular signaling on the control of bovine oocyte nuclear maturation.



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### **Ovarian response of rats and gilts submitted to different commercial equine chorionic gonadotropins (eCG)**

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**Keywords:** bioassay, biological activity, eCG.

Different commercial preparations of equine chorionic gonadotropin (eCG) have been extensively used to induce superovulation or to stimulate follicular growth in cattle submitted to embryo transfer and artificial insemination programs. However, sometimes eCG treatments result in low ovarian response, suggesting differences in the potency of commercial eCG preparations. This study evaluated the biological activity of different commercial eCG products available in Brazil. In the first experiment, four products (A, B, C, and D) from different laboratories were tested in rats using the classical method of Cole and Erway based on gain of ovarian weight. Immature 21–25 day old Wistar female rats received a single sc injection of 10 IU eCG. Saline and eCG from Sigma were used as negative and positive control, respectively. Autopsy was performed 48 h after eCG or saline injection and the ovaries were collected and weighed. Data were analyzed by the Student t test. Ovarian weight (g) from Sigma eCG treated females ( $0.076 \pm 0.013$ ) was similar to females treated with product A ( $0.071 \pm 0.004$ ), C ( $0.076 \pm 0.005$ ) and D ( $0.095 \pm 0.010$ ), however, there was higher ( $P < 0.01$ ) than saline ( $0.033 \pm 0.002$ ) and product B ( $0.038 \pm 0.002$ ). The second experiment was designed to compare the ovarian response and ovulation rate of gilts treated with two commercial eCG (A and B). Eighteen immature gilts (6 per group) received im injections of 0 (Control) or 750 IU eCG (products A and B). Seventy-two hours later, all females received im 500 IU of hCG to induce the ovulations. Animals were slaughtered on day 5 after hCG and evaluated the number of corpora lutea. Data were analyzed by Chi square test. Female treated with the product A had higher (5/6, 83.3 %) ovulation rate than that those treated with the product B (1/6, 16.7%) or Control (0/6, 0.0%) ( $P < 0.01$ ). In conclusion, there are differences in the bioactivity of commercial eCG products and these differences may contribute to the variability of ovarian response. Experiments using animals is a way of assessing the quality of these products. However, considering the ethical and political pressures on the use of laboratory animals, there is a need to develop alternative methods of analysis, such as in vitro and physical chemical assays.

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### **Effects of supplementation of medroxyprogesterone acetate in the pregnancy rate of sheep embryos recipients**

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**Keywords:** embryo, progesterone, sheep.

The embryonic death in early pregnancy may be a limiting factor for commercial production of embryos in sheep production. These animals are corpus luteum dependent in the first third of gestation, with the placenta participating effectively in the production of progesterone from 50-60 days of this phase. Whereas the low concentration of progesterone is related to the high rates of embryo death, it is possible that, by ensuring high levels of this steroid from exogenous applications, there higher rates of pregnancy can be achieved in this species. The objective of this study was to determine the effect of medroxyprogesterone acetate (MAP), administered through intravaginal sponges, on the pregnancy rate in sheep receiving embryos in commercial embryo transfer programs. Sixteen Dorper sheep were used as donors of embryos, and 101 crossbred Santa Inés sheep were used as recipients. Estrus synchronization was performed by applying intravaginal sponges impregnated with 60 MAP (Progespon®) for a period of 11 days, plus a 300 IU equine chorionic gonadotropin (Novormon®) at the time of the removal of the sponges. Superovulation of donors was stimulated with a total of 256mg FSH (Foltropin \_ 1659 ® \_ 1669), administered in smaller doses. Five days after conception, the embryos were collected and transferred. The recipients were randomly distributed into two groups, the Experimental group (GE) and the control group (GC). The pregnancy diagnoses were carried out by ultrasound from 21 and 55 days after the embryo transfer. GE females (n = 53) initially received a sponge with 60 MAP at the time of transfer of the embryos, which was replaced every 11 days, in a total of 5 substitutions. The recipients of the GC (n = 48) did not receive sponges. The results between the groups were compared statistically by the Chi-square test, which differed significantly from each other (P < 0.01) (GE: 85.7% and 55.5% GC of pregnancy rate). This study demonstrated the effectiveness of MAP supplementation on the pregnancy rate in sheep receiving an embryo. It was observed that the administration of the progestin promoted a gain exceeding 30% in pregnancy rate when compared with the control group. This difference is probably due to the fact that the high level of progestin from the intravaginal sponges ensured a uterine environment favorable for embryonic development in the early period of pregnancy, in which period the sheep is corpus luteum dependent for maintaining the pregnancy.



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### **Influence of nitric oxide in the levels of cyclic nucleotides and the resumption of meiosis during in vitro maturation of bovine oocytes**

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**Keywords:** cAMP, cGMP, nitric oxide.

The oocyte maturation in vitro is a limiting factor in the production of embryos. Nitric oxide (NO) acts via the guanylate cyclase (GC) by increasing intracellular levels of cGMP in cumulus-oocyte complexes (COCs). The cGMP in turn, can control the levels of cAMP, which acts in the control of meiosis resumption (Richard, 2007. *J Anim Sci*, 85, 4-6). The objective of this work was to verify the influence of SNAP (S-nitroso-N-acetyl-DL-penicillamine, Sigma, Germany, NO donor) on the levels of cAMP, cGMP and NO and on germinal vesicle breakdown (GVBD) during IVM in cattle. The COCs were aspirated from ovaries obtained from abattoirs, matured (groups of 20 COCs) in droplets (100  $\mu$ l under mineral oil) of IVM medium (TCM199 + 0.25 mM sodium pyruvate, 0.1% PVA and 25  $\mu$ g/ml gentamicin) in an incubator at 38.5° C and 5% CO<sub>2</sub> in air. The COCs were divided into three groups: CONTROL – IVM medium; SNAP - IVM medium with 10<sup>-7</sup>M SNAP and SNAP+ODQ (1H-[1,2,4]oxadiazole [4,3-a] quinoxalin-1-one, Sigma, Germany, inhibitor GC) - IVM medium with 10<sup>-7</sup>M SNAP and 10<sup>-4</sup>M of ODQ. After 9 hours IVM, NO quantification in the medium and the rate of oocyte GVBD (5 replicates) were determined. cAMP and cGMP levels were measured in COCs matured for 1, 2 and 3 hours (3 replicates). GVBD was observed using anti-lamin A / C-DAPI (Prentice-Biensch et al., 2012, *Theriogenology*, 78, 1633-1638). NO quantification was determined using an indirect method by quantifying nitrate (Griess method) using the Griess Reagent System kit (Promega Corporation, Madison, USA) and to measure cAMP and cGMP levels, an enzyme immunoassay was used (EIA cGMP e EIA cAMP kits, Enzo life Sciences, Farmingdale, USA), following the manufacturers' instructions. Data were analyzed by ANOVA followed by Tukey test for GVBD and levels of NO, cAMP and cGMP were analyzed using ANOVA with two criteria followed by the Bonferroni test. The significance level was 5%. GVBD in SNAP (53.4%) decreased ( $P < 0.05$ ) when compared to the other groups (78.4 to 73.4%,  $P > 0.05$ ). Nitrate concentration in SNAP (48.3  $\mu$ M nitrate) was superior ( $P < 0.05$ ) to the others (37.8 and 38.0  $\mu$ M  $P > 0.05$ ). cGMP levels in SNAP at 1 h IVM (4.0 pmol/COC) were similar to immature 0h control (4.4 pmol/COC,  $P > 0.05$ ) and both were superior ( $P < 0.05$ ) to all other groups and time points (1.2 to 3.0 pmol/COC,  $P > 0.05$ ). However, cAMP levels did not differ between the groups at all intervals (0.1 to 0.4 pmol/COC,  $P > 0.05$ ), and all were lower than 0 h IVM (1.0 pmol / COC,  $P < 0.05$ ). In conclusion, the use of NO donor (SNAP) reduces the rate GVBD by increasing the levels of NO and cGMP. The elevation of cGMP by NO, even if only on the first hour of culture was sufficient to delay meiosis resumption, but this effect was not mediated by cAMP pathway. Additional studies are underway to determine the involvement of other factors on the effects observed.

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### **Effect of chemical activation treatments on the development and quality of bovine embryos generated by intracytoplasmic sperm injection (ICSI)**

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**Keywords:** activation, DMAP, ICSI.

Intracytoplasmic sperm injection (ICSI) is an assisted reproductive technique that has been used with considerable success in humans to overcome certain male infertility problems (Palermo et al., 1992, *Lancet*, 340, 17–18). In bovine, however, the efficiency of this technique is far from optimal. Oocyte activation has been recognized as one of the key steps during the ICSI procedure in this species. The objective of the present study was to evaluate the effect of three chemical activation treatments 6-dimethylaminopurine (DMAP), cycloheximide (CHX), and ethanol (ETOH) on the development and quality of bovine embryos generated by ICSI. Cumulus-oocyte complexes were aspirated from abattoir ovaries, selected and matured in 400  $\mu$ l drops of standard TCM-199 maturation medium for 22 h at 38.5°C and 5% CO<sub>2</sub>. ICSI was performed using an inverted microscope with Hoffman optics (Eclipse TS100F, Nikon Instruments Inc., NY, USA) using hydraulic micromanipulators (Narishige International USA). Motile sperm were selected and subjected to tail scoring before being aspirated into the injection pipette. Following breakage of the oolemma, the spermatozoon and the aspirated ooplasm were expelled back into the oocyte. Injected oocytes were randomly assigned to the different activation treatments and cultured in 50  $\mu$ l drops of KSOM medium (culture medium regularly used in our laboratory) under mineral oil at 38.5°C and 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>. Cleavage was recorded at 72 h and blastocysts rate at 192 h. Quality of embryos was assessed by staining with Hoechst and propidium iodide (Fouladi-Nashta et al., 2005, *Reproductive BioMedicine Online*, 10, 497-502). The data were transformed to arcsine, treatment effects were analyzed by ANOVA and means were compared using Tukey's test with Statgraphics Plus 2 Software. Partial results with a total of 246 injected oocytes (95, 104 and 47 for DMAP, CHX e ETOH, respectively) showed differences in cleavage ( $p < 0.01$ ) in DMAP and CHX groups (83 and 72%, respectively), relative to ETOH (20%). Similarly, the rate of blastocysts at 192 h was higher with DMAP and CHX (34 and 21%, respectively), relative to ETOH (5%). Quality of embryos was not different among CHX and DMAP treatments (ETOH was not included in this analysis due to the low number of blastocysts obtained with this treatment). In conclusion, the results show that activation of bovine oocytes after ICSI is more efficient with DMAP and CHX, compared to EtOH, although the quality of the embryos was not different. Studies are underway to establish the effect of these treatments on the ploidy of the embryos.

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A229 Embriology, Biology of Development and Physiology of Reproduction

### **Apoptosis in bovine embryos produced in vitro with different kinetics of development**

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**Keywords:** bovine, development kinetics, embryo.

The kinetics of embryonic development may be related to the viability of IVP embryos, regarding the cellular stress. Although it still remains the hypothesis that embryos presenting higher metabolism could have a higher viability, embryos that develop faster in the beginning also present higher stress response. Based on these data, our goal was to characterize in bovine embryos of different developmental kinetics, the pattern of cell death induced by apoptosis. For that, the cumulus-oocyte complexes were aspirated from slaughterhouse ovaries, selected (grades 1 and 2), placed in drops with 90µl of IVM medium (M-199 with bicarbonate + hormones) (20 oocytes / drop) and cultured for 22 hours at 38.5 ° C and 5% CO<sub>2</sub>. Matured oocytes were fertilized in vitro in 90µl drops of IVF medium (Parrish et al. 1,988 Biol. Reprod. 38th, 1171-1180) at 38.5°C, an atmosphere with 5% CO<sub>2</sub> in air and high humidity for 18 hours. Subsequently, the zygotes were transferred to individual 20µl droplets of culture medium (SOF supplemented with essential and nonessential amino acids, and 5% fetal bovine serum) in a well well system (WOW) (adapted from Feltrin et al., 2006, Proceedings of the 58th Annual Meeting of the SBPC - Florianópolis, SC), and left for 7 days in an incubator with 5% CO<sub>2</sub> in air and high humidity at 38.5 ° C. Embryos were classified as fast (4 cells at 40hpi) and slow (2 cells at 40hpi) and were evaluated for DNA fragmentation (TUNEL, Invitrogen) and the presence of caspase-3 and 7 (Invitrogen) at cleavage (40hpi) and blastocyst (186hpi) stages. TUNEL data were assessed as the relation of TUNEL positive cells / total cells expressed in percentage. Regarding the caspases, embryos were classified according to the intensity of the staining in “high”, “medium, and “no caspase”. The results were analyzed by Student's *t* test from a total of 3 manipulations with at least five embryos per group (Prism 5 GraphPad Inc.). There was no difference between the cleaved embryos regarding the presence of caspase 3 and 7. The group of fast blastocysts showed a tendency for higher caspase compared to slow blastocysts. There was no difference between cleaved embryos (fast: 14.1 ± 10.1; Slow: 3.1 ± 3.1) and blastocyst (fast: 7.2 ± 3; slow: 11.9 ± 2.6) for the number of TUNEL positive cells relative to total cells. These results demonstrate that the different developmental kinetics does not affect embryonic viability in relation to the induction of cell death by apoptosis.

**Acknowledgments:** FAPESP 2012/10351-2 and UFABC.



A230 Embriology, Biology of Development and Physiology of Reproduction

### **Influence of vaccination against IBR, BVD and leptospirosis in the reproductive health in cows in the Amazon region**

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**Keywords:** conception rate, farrowing rate, pregnancy loss.

In previous studies there was a high percentage of cows with positive serology for Bovine Herpesvirus 1 (BoHV-1), Bovine Viral Diarrhea Virus (BVDV) and Leptospira hardjo (Flores et al., 2005, *Pesquisa Veterinária Brasileira*, 25, 125-134; Junqueira et al., 2006, *Ciências Agrárias*, 27, 289-298). This study aimed to evaluate the pregnancy loss from 60 days after FTAI until the moment of the parturition in beef cows from Nelore (n = 4534), vaccinated (n = 2266) or not Nelore breed (n = 2268) for IBR (Infectious Rhinotracheitis Bovine), BVD (Bovine Viral Diarrhea) and Leptospirosis. The work was conducted in the Northern region of Brazil, the city of Boca do Acre - AM, at Nossa Senhora Aparecida farm, where the animals used in the experiment were randomly assigned into two groups. The animals in the group that was vaccinated were vaccinated for IBR, BVD and Leptospirosis (Cattle Master® + 4 L5, Pfizer, São Paulo-SP) 5 mL intramuscularly, the first dose was given at the beginning of the FTAI protocol (day 0) and the second dose at diagnosis of pregnancy (day 30). To avoid statistical bias the same estrous cycle handling protocol was used between the groups, with a duration of 11 days, where on Day 0 (D0) the intravaginal device releasing progesterone (P4) was introduced, which is unused (CIDR®, Pfizer, São Paulo-SP) and the application of 2 mL of estradiol benzoate, IM (2,0 mg estradiol benzoate, Estrogin®, Farmavet, São Paulo-SP), after seven days was applied PGF2α, IM (12.5 mg de Dinoprost, Lutalyse®, Pfizer, São Paulo-SP). On day 9, the CIDR® was withdrawn, and 0.5 mg estradiol cypionate injected IM (E.C.P.®, Pfizer, São Paulo-SP) and the calves were removed for 48 hours (Shang). All animals were inseminated 46 to 52 hours after CIDR®. The inseminations were performed by the same inseminator. Miscarriages were evaluated from 60 days after FTAI until parturition. Miscarriages were calculated by subtracting the total of pregnant females at 60 days from the numbers of calves born. Data were analyzed by Chi-square. Conception rates did not differ (p = 0.19) between vaccinated and unvaccinated cows [53.57% (1215/2268) vs. 56.48% (1280/2266), respectively]. The birth rate did not differ (p = 0.23) between vaccinated and unvaccinated cows, [92.18% (1120/1215) vs. 95% (1216/1280), respectively]. The rate of pregnancy loss differed (p = 0.02) between vaccinated and unvaccinated cows, [7.81% (95/1215) vs. 5% (64/1280), respectively]. We concluded that females vaccinated for IBR, BVD and Leptospirosis have lower pregnancy losses between 60 days after FTAI and parturition.



A231 Embriology, Biology of Development and Physiology of Reproduction

### **Functional ultrasound characteristics of corpus luteum during the first weeks post-insemination in pregnant and non-pregnant beef cows**

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**Keywords:** corpus luteum, pregnancy diagnostic, progesterone.

Pregnancy diagnostic methods that detect non-pregnant cows before the following ovulation ( $\approx$  day 21-post-ovulation) are needed to reduce the interval of inseminations (AI) in beef cattle. This study aimed to: (1) identify functional characteristics of corpus luteum in pregnant and non-pregnant cows during early gestation in beef cattle; and (2) determine the accuracy of detection of non-pregnant animals through the evaluation of luteal function between 18 to 22 days post-AI. Nelore cows ( $n=27$ ) were submitted to timed-AI after synchronization of ovulation. Doppler and B-mode ultrasonography and plasma progesterone (P4) concentrations were measured on days 8, 12, 15, 18, 20, 22 and 30 post-AI for ovulated cows ( $n=22$ ). In each ultrasonography exam the dimensions and peripheral and total blood flow of the CL, and presence of the embryonic vesicle were evaluated. Loss of pregnancy was detected based on the occurrence of structural luteolysis, that was defined by the following two criteria: A) reduction in  $\geq 25\%$  of luteal area on days 18, 20 or 22 compared to day 8 post-AI; or B) CL with area  $< 2\text{cm}^2$  and blood flow  $\leq 25\%$  of total CL area. Repeated variables were analyzed by split-plot ANOVA using the PROC MIXED procedure (Version 9.2; SAS Institute), considering the effects of group (pregnant and non-pregnant), day and interaction. Pregnancy was diagnosed in 45.5% (10/22) of cows on days 25 and 30 post-AI. An effect of group, day and their interaction was detected in all variables ( $P < 0.05$ ). There was a reduction in the mean P4 concentration and CL area in the non-pregnant group starting on day 20 post-AI ( $P < 0.05$ ). CL diameter and volume were reduced in non-pregnant cows on day 18 post-AI, and were greater on day 12 post-AI in pregnant cows ( $P < 0.05$ ). Luteal blood flow (peripheral and total) was greater on days 8, 12, 20, 22 and 25 post-AI in the pregnant group than in the non-pregnant group ( $P < 0.05$ ). Although P4 concentrations did not differ ( $P > 0.05$ ) on day 12 post-AI, the greater vascularization and volume of CL on pregnant cows indicated a greater luteal development, which may favor maintenance of pregnancy. Based on the two criteria used to diagnose non-pregnant cows, 25% (3/12), 66.7% (9/12) and 91.7% (11/12) of non-pregnant cows were detected by criteria A, and 25% (3/12), 91.7% (11/12) and 91.7% (11/12) by criteria B, on days 18, 20 and 22, respectively; no false-negative diagnostic was observed. The results suggested that CL of pregnant beef cows are larger and more vascularized on day 12-post AI. In conclusion, the detection of structural luteolysis by ultrasonography on days 20 and 22 post-AI is a reliable tool for the early diagnosis of non-pregnant Nelore cows.

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A232 Cloning, transgenesis and stem cells

### **Expression of molecular markers for bovine spermatogonial stem cells in prepubertal and adults Nelore**

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**Keywords:** molecular marker, bovine, Spermatogonial stem cell.

The little knowledge about expression of molecular markers for bovine's Spermatogonial Stem Cells (SSCs) and the negative effect of ageing over these cells (Kokkinaki et al., 2010, Reproduction, 139, 1011-20) reduce the sorting efficiency by flow cytometry and immunocytochemistry. The goals of this study were to evaluate and to compare the expression of SSCs molecular markers by flow cytometry (SSEA4) and by immunocytochemistry (SSEA4, PGP 9.5 and alpha-6-Integrin (A6Int)) in cells from prepubertal (aged of 5 months, n = 10) or adults Nelore males (aged 3-4 years age, n = 10) before and after differential plating. In order to perform the experiment, biopsies from testicular parenchyma were minced and digested with collagenase (1mg/ml, 30min at 37°C followed by trypsin (2.5mg/ml, 5 min at 37°C). Viable cells were plated on cell culture dish (100mm) previously covered with bovine serum albumin (0.5 mg/ml) and cultured overnight in high humidity atmosphere with 5% of CO<sub>2</sub> at 37°C. Viable cells from the supernatant were fixed with cold ethanol 70% and incubated with antibody anti A6Int labeled with Alexa Fluor 488 (BioLegends®, San Diego, CA, USA), antibody anti PGP 9.5, anti SSEA4, anti Vimentin or anti Cytokeratin (all antibodies from Abcam®, Cambridge, MA, USA). Samples previously incubated with antibody anti PGP 9.5 and SSEA4 were also incubated with a second antibody labeled with FITC (Abcam®, Cambridge, MA, USA) whereas samples previously incubated with antibody anti Vimentin and Cytokeratin were incubated with secondary antibody but labeled with Texas Red Sulfonil Chloride (Abcam®, Cambridge, MA, USA). Percentage of positive cells for SSEA4 was determined by flow cytometry (Attune, Applied Biosystems, Foster City, CA, USA) and the immunocytochemistry evaluation were performed by fluorescence microscope (Olympus IX-81, Olympus, Tokyo, Japan). Flow cytometry data was analyzed by FlowJow (Tree Star, Ashland, OR, USA) and Wilcoxon statistical test was performed (STATA, College Station, Texas, USA). Immunocytochemistry results strongly suggest that SSEA4 is a molecular marker for bovine SSCs. Positive staining also was observed for PGP 9.5, A6Int, Vimentin and Cytokeratin. SSC purification by differential plating was not as efficient as expected because positive staining for vimentin was observed after the treatment. This result suggests that there is a subpopulation of Sertoli cells after the purification. Furthermore, no effects of differential plating and ageing were observed on percentage of positives SSCs for SSEA4 by flow cytometry. Thus, the expected high number of SSEA4 positive cells after the cell purification was not confirmed. In conclusion, SSEA4 is a potential molecular marker for bovine SSC and the purification of SSCs by overnight differential plating with bovine serum albumin treatment was not as efficient as expected.

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A233 Cloning, transgenesis and stem cells

### **Cryopreservation of mesenchymal stem cells derived from bovine adipose tissue with propylene glycol or DMSO**

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**Keywords:** DMSO, Mesenchymal stem cells, propylene glycol.

Cryopreservation is a strategy used for the conservation of cells for extended periods, however, it is important to maintain their characteristics during this process. The use of DMSO as cryoprotectant for stem cells is controversial because it can potentially interfere in the undifferentiated condition of these ones. The objective of this study was to evaluate the use of Propylene Glycol (PG) as an alternative to Dimethylsulfoxide (DMSO) in the cryopreservation of mesenchymal stem cells (MSCs) obtained from bovine adipose tissue. For this, a sample of adipose tissue was collected from the tail base of a female bovine and submitted to enzymatic digestion with collagenase (0.075%). The isolated cells were grown in DMEM supplemented with 10% FCS, at 37°C and 5% of CO<sub>2</sub>, and after reaching 70% confluence part of the cells were maintained in culture as a control, and also used for induction of osteogenic differentiation by the MSC Osteogenic Differentiation Medium kit (Lonza Pharma), while another part was used for cryopreservation. For this, the cells were trypsinized, centrifuged and resuspended in medium containing 10% FBS and 10% cryoprotectant (DMSO or PG) at a concentration of  $1 \times 10^6$  cells/mL. Freezing was done in cryotubes at -1°C/min with the Mr. Frosty® apparatus (Nalgene Nunc Cooler, USA) at -80°C and then kept at -196°C. For thawing, cryotubes were placed in the water bath at 37°C, and then the content was transferred to a tube containing 2 ml of DMEM with 10% FCS, centrifuged and resuspended in 500 µL of culture medium. One aliquot was used to determine the immediate survival by trypan blue staining. Of the remainder, 10000 cells were cultured in 1.9 cm<sup>2</sup> wells, in triplicate, and counted after 24, 48 and 72 hours to evaluate the growth curve. Other aliquots were used to perform the Comet assay (Collins, 2004, Mol. Biotechnology, 26, 249-261) and to the induction of differentiation. Immediately after thawing, the survival rate was 85.0% and 68.8% for cells frozen with DMSO and PG, respectively. The cells submitted to growth curve showed no statistical difference between treatments (DMSO x PG) relative to the number of cells considered viable in 24 h (25,667 x 18,667) and 48 h (40,500 x 42,833), however, with 72 h the number of cells was significantly higher (79,333) with DMSO treatment than with PG (67,167). The cells cryopreserved with PG showed higher DNA integrity (69.5%), assessed by the Comet assay, while only 46.5% of cells cryopreserved with DMSO didn't show any damage (p=0,05). The induction of differentiation into osteogenic tissue was positive in all groups, showing their multipotency. Therefore, despite the PG shows lower recovery of viable cells, it promotes low damage in the genetic material of cells and can be used as an alternative to DMSO.



A234 Cloning, transgenesis and stem cells

### **Pregnancies and births of bovine cloned embryos from amniotic fluid and adipose tissue cells collected in vivo**

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**Keywords:** Bovine, embryos, nuclear transfer.

Cells from amniotic fluid (CAF) and adipose tissue (CAT) were characterized as mesenchymal stem cells in some animal species (Mauro et al., 2010, *Vet Res Commun* 34, S25-S28; De Mattos Carvalho et al., 2009, *Vet Immunol Immunopathol*, 132, 303-06), and despite its potential for use as nucleus donors, it has not been used in nuclear transfer (NT) in bovine. The objective of this study was to test the efficiency of use of CAF and CAT in NT. The recovery of CAFs were performed by ultrasound-guided intra vaginal aspiration, without damaging the 64-days fetus. The amniotic fluid was centrifuged and the cells cultured in Amniomax Complete II medium (Gibco, Rockville, USA). CATs were collected by perineal biopsy of the same calf with seven months old and explants were cultured in DMEM (Invitrogen Life Science, USA). The morphological characterization was performed by Scanning Electron Microscopy. The isolated cells were used in the NT procedure according Kuroiwa et al. (2002, *Nature*, 20, 889-894), with modifications. The statistical analysis was performed by ANOVA and Tukey test ( $p < 0.05$ ) (SAS 9.1.2). The blastocyst rate based on number of cleaved structures was  $45.46 \pm 13.03$  and  $46.47 \pm 7.92\%$  for CAF and CAT, respectively, with no significant difference. The pregnancy rate at 35 days was 12.5% (1/8) to CAF and 25% (1/4) to CAT. Hydropsy in pregnant recipient with CAF was observed at 245 days. The birth was induced at 277 days and cesarean section was performed after 36h to remove the calf alive. The animal weighed 58.5 kg and on the external examination was observed teeth not completely developed, thick umbilical cord and bilateral flexion of the metatarsophalangeal joints. Hydrothorax serous, fluid throughout the trachea, fluid in the lung parenchyma, enlarged liver with yellowish and fat deposition in the surface were observed in the necropsy. The cloned calf from the CAT was born at 291 days with 35 kg without birth complications. No clinical alteration was observed in this calf, which is still healthy. The pregnancy rates obtained in this study were higher than those reported in the literature, however, the calf derived from CAF presented the Large Offspring Syndrome (LOS), frequently associated with cloned animals, whereas LOS was not observed in calf from CAT, suggesting that this new cell type can be used in bovine NT but further studies should be carried out.



A235 Cloning, transgenesis and stem cells

### **Generation of bovine induced pluripotent stem cells (biPS) and production of cloned embryos derived from biPS cells after nuclear transfer**

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**Keywords:** induced pluripotency (iPS), Nuclear transfer; stem cells.

Bovine embryo production through nuclear transfer (NT) is still hampered by low developmental rates, due to the incomplete reprogramming of the nuclei donor. Strategies used to promote a better nuclei reprogramming, for example, the use of more undifferentiated cells as nuclei donors have already showed interesting perspectives. It is known that *in vitro* maintenance of pluripotency in bovine embryonic stem cells-like is difficult, therefore, the present study aimed to generate bovine induced pluripotent stem cells (biPS) and use them as nuclei donors in NT, enhancing therefore the efficiency of nuclei reprogramming. Bovine iPS cells were produced through lentiviral transduction of murine transcriptional factors related to pluripotency (Oct4, Sox2, c-Myc and Klf4 - OSKM) in bovine fibroblasts. Cells were characterized regarding morphology, gene expression, immunofluorescence of pluripotency factors, alkaline phosphatase detection, embryoid body formation, *in vitro* differentiation, *in vivo* teratomas formation and subsequently used as nuclei donor in NT. Briefly, bovine oocytes obtained from slaughterhouse ovaries were *in vitro* matured for 18h, enucleated and reconstructed with biPS (n=203) or bovine fetal fibroblasts (bFF, n=153), in five repetitions. After reconstruction, zygotes were activated with ionomycin and 6-DMAP and *in vitro* cultured until blastocyst stage. Fusion, cleavage (48h after activation) and blastocyst (192h after activation) rates were evaluated and results were submitted to Chi-square test at 5% de significance. No differences were observed between groups regarding cleavage (81.53 vs 88.96%) or embryo production (22.68 vs 29.63%, respectively), however embryos reconstructed with biPS cells presented a reduced fusion rate (47.78 vs 70.59%). In conclusion, biPS cells were produced and derived cloned embryos after NT. A better understanding of nuclei reprogramming mechanisms and production of animals derived from reprogrammed cells should lead to enhance the efficiency of reproductive biotechnologies.

**Financial support:** FAPESP and CNPq.



A236 Cloning, transgenesis and stem cells

### **Co-culture of bovine embryos with adult stem cells derived from adipose tissue (preliminary results)**

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**Keywords:** co-culture, embryo, stem cells.

Somatic cells used in co-culture of embryos have the ability to remove components harmful to embryonic development, such as reactive oxygen species (ROS), (Mouatassim S. et al., 2000, Eur J Obstet Gynecol Play Biol, 89, 1-6). Stem cells from adipose tissue (SCAT) have the potential for adipogenic, osteogenic, chondrogenic and myogenic differentiation (Zuk et al., 2002, Mol Biol Cell, 13, 4279-95). Nowadays, these cells can be considered as one of the best sources of stem cells for various types of clinical studies, because it is easily isolated and exhibit multipotency (Zhu et al., 2008, Cell Biochem Funct, 26, 664-75). Thus, the objective of this study was to evaluate the use of SCAT in co-culture of bovine embryos, aiming to improve the protocol for in vitro embryo production. The SCAT were obtained from fat of cattle from slaughterhouse, isolated with collagenase type I (0.001 g/ml) for 180 minutes and cultured in IMDM (Iscove's Modified Dulbecco's Medium, Gibco®) supplemented with 10% fetal calf serum and gentamicin (0.01 g/ml). To assure the multipotency of SCAT, cells were differentiated on passage P7 in osteoblasts (confirmed by staining with 2% of alizarin red S), adipocytes (staining with 25% Oil Red) and chondrocytes (staining with Alcian Blue 1 %) with STEMPRO Differentiation Kit Gibco®. Cattle's cumulus-oocytes complex (Cocs) were matured in vitro in TCM-199 medium supplemented with 10% FCS, FSH and LH, for 20 hours, in 38.5°C and 5% CO<sub>2</sub>. The Cocs were fertilized in Talp-hepes FERT medium supplemented with heparin, penicillamine, hipotaurine, epinephrine and BSA, and grown under the same conditions cited for the IVM. After 24 hours of fertilization, the zygotes were distributed to droplets of SOF medium supplemented with BSA (6 mg/ml) and 10% FCS, in the following experimental groups: SOF without monolayer (SOF), SOF with granulosa cells (SOF-G), SOF with one thousand SCAT (SOF-ONE THOUSAND) and SOF with ten thousand SCAT (SOF-10 THOUSAND). The cleavage rate was evaluated on the 2nd day of culture and the blastocyst formation on day 7, and the results were analyzed by ANOVA using Bonferroni post-test, adopting the significance level of 5 %. Regarding to cleavage rate, there was no difference ( $p > 0.05$ ) between the groups analyzed, however, the group SOF-10 THOUSAND ( $n = 61$ ) increased significantly ( $p < 0.05$ ) the production of blastocysts in comparison to groups SOF-G ( $n = 59$ ) and SOF ( $n = 63$ ) at the end of the 7th day of culture ( $47.20 \pm 1.46$  vs.  $31.43 \pm 1.49$  vs.  $19.15 \pm 1.29$ , respectively), though it did not differ ( $p > 0.05$ ) from SOF-ONE THOUSAND group ( $32.73 \pm 1.16$ ;  $n = 61$ ). These preliminary results show that the use of SCAT significantly increased the rate of bovine embryonic development to the blastocyst stage, which may be due to its effect against ROS in comparison to traditional co-culture with granulosa cells.



A237 Cloning, transgenesis and stem cells

### **Ovarian follicle-like structures differentiated in vitro from pig skin derived fibroblasts treated with 5-azacitidine**

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**Keywords:** 5-azacitidine, differentiation, fibroblasts.

This study aimed to evaluate the effects of 5-azacitidine (5-Aza) on the expression of mRNA for Oct-4, Nanog, Rex-1 and Sox-2 in pig fibroblasts, and to investigate the influence of follicular fluid (FF) associated or not with bone morphogenetic protein-4 (BMP-4) on differentiation of treated fibroblasts into ovarian follicle-like structures after 21 days of culture. To this end, fibroblasts isolated from skin biopsies were cultured in DMEM supplemented with 1 $\mu$ M 5-Aza, 20% FCS, penicillin and streptomycin in an incubator with 5% CO<sub>2</sub> at 37°C for 18 h. Then, the fibroblasts were cultured in DMEM supplemented with 5% FCS, 0.23mM sodium pyruvate, 0.1mM nonessential amino acids, 2mM L-glutamine, 0.1mM  $\beta$ -mercaptoethanol, 100 IU/mL penicillin and 100 mg/ml streptomycin (control medium). For the treatments, the cells were cultured in control medium supplemented with 5% porcine FF or both 5% FF and 50 ng/mL BMP-4 (Sigma). As a control, fibroblasts that were not treated with 5-Aza were cultured in control medium. Besides evaluating cell morphology after each period of culture (4, 7, 14 and 21 days), the expression of markers for pluripotent cells (Oct-4, Nanog, Rex-1 and Sox-2), primordial germ cells (Dazl and Vasa) and oocytes (GDF-9B) was evaluated by qualitative PCR. After 5 to 7 days of culture, several colonies of round cells in the three different media tested were observed, and some of these cells had a diameter of approximately 25 $\mu$ m. The morphology of these cells resembled that of oocytes before primordial follicles formation. After 14 to 21 days in culture, primordial and primary follicle-like structures, with a diameter of 40-50 $\mu$ m were observed. These structures had a large centrally localized cell surrounded by small cells, being the morphology similar to that of oocyte and granulosa cells. The PCR showed that 5-Aza induced the expression of mRNAs for Oct-4, Nanog, Rex-1 and Sox-2 and the cells continued to express Nanog and Sox-2 for up 21 days of culture in the three tested media. Expression of Oct-4 and Rex-1 persisted for up 7 days after cultured in all media, but after 7 and 14 days, the expression was kept only in medium with both FF and BMP-4. Regarding to primordial germ cell and oocyte markers, transcripts for VASA and GDF-9B were detected from 4 to 21 days of culture in all tested media. In addition, the expression of DAZL was observed in cells cultured either in control medium for 7 days or in medium with FF and BMP-4 for 14 and 21 days. In conclusion, 5-Aza stimulated the expression of Oct-4, Nanog, Rex-1 and Sox-2 in pig fibroblast and these cells were able to differentiate into ovarian follicle-like structures after 21 days of culture.

**Financial support:** CAPES.



A238 Cloning, transgenesis and stem cells

### **Equine induced pluripotent stem cells (iPS) production through exogenous expression of human factors related to pluripotency**

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**Keywords:** equine, iPS, STEMCCA.

Induced reprogramming has been used as a tool to in order to reprogram somatic cells to a pluripotent state similar to the embryonic stem cells (ESC). Transcription factors previously described by TAKAHASHI and YAMANAKA (Cell, 2006, 126, 663-76), that, when associated are capable of reprogramming cells into a pluripotent state, turning them into induced pluripotent stem cells (iPS), were used in this study. Aiming equine iPS cells (eiPS) production, human factors related to pluripotency OCT4, KLF4, SOX2 and c-MYC in a polycistronic lentiviral vector STEMCCA (stem cell cassette) were introduced in mesenchymal cells derived from equine adipose tissue and cultured at 38.5°C with 5% CO<sub>2</sub> atmosphere and maximum humidity. eiPS cells were characterized regarding their morphology, alkaline phosphatase expression, gene expression, immunofluorescence, embryoid body (EB) formation, spontaneous differentiation *in vitro* and teratoma formation *in vivo*. A non-transduced mesenchymal cell line was used as control group. Equine mesenchymal cells presented colony formation three days after exogenous gene insertion in their genome. These cells were individually cultured after approximately 15 days after transduction and three eiPS cells lines were studied. All three cell lines were tested and presented alkaline phosphatase expression. OCT4 and SOX2 expression was proven by quantitative gene expression and presence of OCT4 protein was confirmed by immunofluorescence for all cells studied. Two cells lines were tested regarding *in vitro* EB formation and both showed efficient generation of EBs. *In vitro* spontaneous differentiation analysis was tested in one cell line, however, it was still possible to observe a high ratio between nucleus/cytoplasm, even after 20 days in culture in fibroblast culture medium, indicating the possibility that full reprogramming and consequent transgene silencing did not occur resulting in continuous exogenous expression of pluripotent factors in these cells. Also, no teratoma formation was observed in a 35 days period after inoculation. Thus, it was possible to demonstrate equine iPS colony formation induced by the introduction of human transcription factors during this study. Studies regarding inactivation of introduced genes and reactivation of endogenous genes, characterizing full reprogramming of these cell lines are still needed and will provide important information about the nuclear reprogramming process for the equine species.



A239 Cloning, transgenesis and stem cells

### **Epigenetic marks and imprinted genes expression in bovine cells**

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**Keywords:** epigenetics, imprinting, reprogramming.

The obtainment of appropriated cellular reprogramming is a hurdle in animal cloning. Many fetal abnormalities found in cloned animals have been correlated to abnormal epigenetic reprogramming of the donor cells. In this context, the disrupted expression of imprinted genes has been highlighted as one of major factor that lead to this increased occurrence of fetal abnormalities such as the large offspring syndrome. Chromatin modifying agents, such as trichostatin-a (TSA), which is an inhibitor of histone deacetylases, have been employed to alter the epigenetic status of donor cells and evaluate the epigenetic mechanisms controlling imprinted genes and its impacts after nuclear transfer. To understand how the TSA can modulate IGF2R expression, we treated non-confluent bovine fibroblast with different concentration of TSA (0.05; 0.25; 1.25 and 6.25  $\mu$ M for 8, 12 and 24-20 hours of culture). Three bovine fibroblast cell lines were treated and evaluated for cell viability by MTT analysis; global expression of IGF2R by qPCR and global di-methylation levels of lysine 9 of histone protein 3 (H3K9me2) by western blotting. The treatment of 6.25  $\mu$ M of TSA decreased cell proliferation and there was a significant decrease of cells proliferation with the time. The TSA did not change global expression of IGF2R in any tested concentration and/or time; however there was a decrease of H3K9me2 levels when cells were treated with 6.25  $\mu$ M TSA for 8 h. Moreover, there was depletion of H3K9me2 levels in the cells treated with 0.25; 1.25 and 6.25  $\mu$ M TSA but not 0.05  $\mu$ M after 24 h of TSA treatment. The qualitative western blotting analysis show an increase of H3K9me2 levels in cells treated for 24 h when compared to those treated for 8 h. Despite alterations of H3K9me2 levels and increased levels of 5-hidroxymethylcytosine by TSA have also been previously reported by others (Perecin et al., 2012, *Reprod Domest Anim*, 47, 503) IGF2R expression remained unaltered, indicating the lack of direct effect of histone methylation levels and 5-hmC levels on IGF2R epigenetic control. The results of this research will contribute to the understanding of the epigenetic mechanisms controlling IGF2R expression.

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A240 Cloning, transgenesis and stem cells

**Birth of an extremely lighter-than-normal asymmetric cloned Gujarat calf at term: similarity to the pathophysiology of intrauterine growth restriction (IUGR) syndromes in humans**

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**Keywords:** abnormal offspring syndrome, IUGR, SCNT.

The *in vitro* production of bovine embryos by *in vitro* fertilization or nuclear transfer procedures is often associated with developmental abnormalities that appear to interfere with the pattern of fetal and placental growth and life *ex utero*, in a set of symptoms collectively called Abnormal Offspring Syndrome (AOS). One of the most common symptoms of the syndrome is the birth of unusually large calves with lower postnatal survival. However, smaller-than-normal calves may also occur on occasions, at a low frequency (Meirelles *et al.*, 2010, *Reprod Fertil Dev*, 22, 88-97). Disturbances in placentation caused by faulty epigenetic reprogramming may at least partly cause changes in the pattern of fetal growth during pregnancy. In human and sheep, nutrient restriction commonly related to reduced placental development or insufficiency is associated with intrauterine growth restriction (IUGR), which may lead to a pattern of asymmetric fetal growth. In such extreme cases, fetal asymmetry is characterized by an increase in brain-to-liver ratio and in abnormalities in internal organs (Cox & Marton, 2005, *Best Practice & Research: Obstetrics & Gynaecology*, 6, 751-64). In this report, we describe a case of fetal growth asymmetry in cattle and the birth of an exceedingly light asymmetric calf after the transfer of cloned embryos. Bovine cloned embryos were produced by Handmade Cloning (HMC) procedures, according to Ribeiro *et al.* (2009, *Cloning Stem Cells*, 11, 377-86), using skin fibroblast cells from three Gujarat females, two from adult and one from neonatal origin. A total of 56 out of 73 (72.6%) structures fused following embryo reconstruction, with 50 cloned embryos *in vitro*-cultured in aggregates (2 x 100%) in the WOW system. Cleavage and blastocyst rates on Days 2 and 7 of development were 92% (23/25) and 32% (8/25), respectively, on a per WOW basis. Eight cloned blastocysts were transferred to six synchronous Holstein-crossed female recipients, resulting in one pregnancy, diagnosed on Day 30 of gestation by ultrasonography. The viable pregnancy, developed from a grade 3 blastocyst stage (stage 6) embryo, was monitored monthly by ultrasonography, with signs of retarded fetal development observed as early as on Day 90 of gestation. Following the pre-induction for parturition on Day 284 of pregnancy, by the administration of 8 mg triamcinolone acetonide (IM), the recipient female calved vaginally on Day 287, delivering a female Gujarat calf weighting 2.2 kg, more than 90% less than the mean birth weight (BW) for the breed (25-30 kg). The calf died soon after birth, and atelectasia was detected after necropsy. The newborn calf was meconium-stained, with a normal coat color and no skin defects. Also, an increased head-to-liver size, bragnatism, lens opacity, apparent arthrogyposis, thicker heart walls, and a poor skeletal muscle development were observed, with underdeveloped internal organs, including kidneys with thinner cortex and a smaller liver. Fetal membranes (FM) were lighter (530 g) and had a smaller cotyledonary surface area (CSA, 366.6 cm<sup>2</sup>) for the species and breed, with fewer than normal cotyledons (n=27) and an abnormal BW-to-FM weight ratio (24.1%) and BW-to-CSA (16.7%), indicating an altered development of the placental tissue in prenatal development. The FM vasculature appeared to be underdeveloped. Similar to the case study, head sparing, i.e., growth of the head at normal or near-normal rate, is usually detectable in cases of IUGR in humans, which appears to be a protective mechanism to ascertain brain development, as placental is insufficient to provide the necessary nutrients to the fetus at mid- to late gestation. The understanding of mechanisms of prenatal growth in normal and abnormal development may be of significance for prevention or attenuation of abnormalities of common occurrence in cattle, being also a potential model for the study of pathophysiological processes related to IUGR syndromes in humans, sheep and other species.



A241 Cloning, transgenesis and stem cells

### **Intracytoplasmic sperm injection (ICSI) mediated transgenesis using bovine sex-sorted semen**

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**Keywords:** ICSI, sex-sorting, transgenesis.

During the production of sex-sorted spermatozoa, the gametes are exposed to damages that reduce the number of viable spermatozoa (Boe-Hansen et al., 2005, *Theriogenology*, 63, 1789-802) but that could be beneficial for transgenesis. Since ICSI technique requires low numbers of sperm, and allows the use of low quality or even death sperm (Goto K et al., 1990, *Vet Rec*, 127, 517-20), its application could increase the efficiency of the use of sex-sorted semen, which usually has a high cost. The objective of this work was to test sex-sorted sperm for ICSI mediated transgenesis. COCs were collected from slaughtered cow ovaries and in vitro matured for 21 h. The sorted Y, X and non sorted semen from the same bull were thawed and co-incubated with 50 ng/ $\mu$ l of pCX-EGFP plasmid for 5 min and used for ICSI (ICSI-Y, ICSI-X and ICSI-NS groups respectively). Injected oocytes were activated by a 4 min exposure to 5  $\mu$ M ionomycin, placed on TCM-199 for 3 h and subsequently treated with 1.9 mM DMAP for 3 h. Sham controls were injected with 50 ng/ $\mu$ l pCX-EGFP. Haploid and diploid parthenogenetic controls were also included (Haplo PA and Diplo PA groups respectively). Embryos were cultured in SOF medium. Cleavage and blastocyst rates were evaluated on Days 2 and 7 post ICSI, respectively. *EGFP* expression was assayed at day 4 and at the blastocyst stage. Differences among treatments were determined by Fisher's exact test ( $P \leq 0.05$ ). Cleavage rates of ICSI-Y (83%, n=106), ICSI-X (83.2%, n=101), Sham (85.3%, n=116) and Haplo PA (76.1%, n=88) were lower than those of the Diplo PA control (95.6%, n=157), and higher than ICSI-NS (50.9%, n=106) ( $P \leq 0.05$ ). Transgene expression levels at day 4 were significantly higher for ICSI-Y (36.4%) and ICSI-X groups (38.1%) than those of ICSI-NS group (29.6%); and all of them differed from Sham control (1%) ( $P \leq 0.05$ ). Although all the groups showed lower blastocyst rates than Diplo PA control (45.9%), only ICSI-NS (9.3%) did not differ from Haplo PA control, which did not produce any blastocyst. ICSI-Y and ICSI-X blastocyst rates (15.9 and 13.1% respectively) were higher than Haplo PA control group, but did not differ from Sham control (9.1%) ( $P \leq 0.05$ ). The percentage of *EGFP* expressing blastocysts did not vary between ICSI groups (36.4; 28.6 and 40% for ICSI-Y, ICSI-X and ICSI-NS respectively), being all of them different from the Sham group (0%) ( $P \leq 0.05$ ). To our knowledge, this is the first report of ICSI mediated transgenesis using sex sorted semen in bovine, and it demonstrates its utility to produce transgene expressing blastocysts with the same efficiency than non sorted semen. It could be a useful strategy to produce transgenic animals of the desired sex, avoiding the problems carried by somatic cell nuclear transfer.



A242 Cloning, transgenesis and stem cells

### **Evaluation of cell viability and gene expression of stress and apoptosis in bovine fibroblasts exposed to ethanol extract of *Azadirachta indica***

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**Keywords:** cell stress, nuclear reprogramming, somatic cell nuclear transfer.

Somatic cell nuclear transfer (SCNT) is an important biotechnological tool, but it still faces many challenges. The synchronization of donor cells has been described as one of the main factors required to achieve a correct nuclear reprogramming (Oback and Wells, 2004, Cloning Stem Cells, 4, 169-174). It has already reported that ethanolic extract of the *Azadirachta indica* (Neem) was efficient to inhibit the cell cycle of bovine fibroblasts (Rabelo et al., 2011, Acta Sci Vet, 39, 338) and that its effect is reversible (Rabelo et al., 2012, Animal Reprod, 9, 652). It is required, however, that the cells remain viable after exposure to the extract in order to support the embryonic development after SCNT. The objective of this study was to evaluate cell viability and expression of stress and apoptosis genes in bovine fibroblasts exposed to ethanol extract of Neem. The cells were cultured in DMEM + 10% fetal calf serum (FCS) and exposed to 100 and 200µg/mL of the extract for 24h. Three repetitions were performed in triplicate. Simultaneously, a serum starvation group (DMEM +0.5% FCS for 72h, without the extract) and a control group (DMEM +10% FCS) were prepared. Cell viability was evaluated by Trypan blue (0.1%) staining, which readings were performed by the Cedex XS Analyser automatic cell counter (ROCHE, Switzerland). Data was analyzed by analysis of variance and means compared by Student Newman Keuls test. Relative quantification of transcripts of stress and apoptosis (HSP70.1A, HSBP1, HSP27.P1, BAX, BCL-2 e WNT5A) was performed by Real-Time PCR and results compared by the comparative Ct method, using the β-actin gene as endogenous reference and control group as calibrator. Data was analyzed by the REST® software using the Pair Wise Fixed Reallocation Randomisation TEST®. The products obtained were also subjected to electrophoresis on agarose gel. Values are shown as mean ± SEM. There was no difference (p>0.05) for cell viability among serum starvation (51.78±3.04%), control (51.04±0.9%) and treatment with 100µg/mL *A. indica* (50.15±1.64%). However, cell viability decreased (p<0.05) when cells were exposed to 200µg/mL (45.1±2.76%). There was a decrease (p<0.05) on expression of genes associated to stress and apoptosis in cells exposed to *A. indica*, except for HSP27.P1 gene in 100µg/mL and HSP70.1A gene in 200µg/mL extract, which expression were not altered. These results indicates that 100µg/mL ethanol extract of *A. indica* can be a complement for in vitro cell culture, keeping cell viability and with a potential protective effect against stressful conditions of *in vitro* culture. Taking together, the previous results (Rabelo et al, 2011 and 2012) and the present one suggest that ethanol extract of *A. indica* can be an alternative for synchronizing cells donor nuclei.

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A243 Cloning, transgenesis and stem cells

### **Clinical findings and neonatal care of two transgenic cloned calves containing coagulation factor IX: a case report**

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**Keywords:** bovine, newborn care protocol, SCNT.

Both basic and applied research benefit from the technology of transgenic animal production. The production of transgenic animals through nuclear transfer (NT) presents advantages over other methods, in special, the possibility to select a transgenic donor cell prior cloning and therefore to produce an homogenous herd without mosaic offspring. Despite all efforts, the neonatal cloned calves mortality is still high, approx. 50%. The pulmonary hypertension is a problem that requires major clinical attention, representing the neonate disability on adapting to the new environment conditions. This study aimed to support the extra uterine life of two transgenic cloned calves by using neonatal care protocol focused on pulmonary function improvement. Bovine epithelial mammary cells, transduced with lentivirus harboring coagulation factor IX (FIX) driven by  $\beta$ -casein promoter, were submitted to NT. From 21 embryos transferred into recipient cows, two pregnancies were maintained until 290 days. For induction of parturition, 25mg (IV) dexamethasone (DEX) were used. Once no cervical dilatation was observed, the two calves were obtained by cesarean, 44h after DEX application. Both calves weighted approximately 40kg. At delivery, both newborn had their upper airway aspirated to remove excess fluid, for about 10 minutes. One minute after birth, both animals presented low APGAR scores, which is a manner to measure the newborn vitality by heart rate, respiratory rate, response to stimulation and mucous coloration; animal 1 (FIX1) presented APGAR score 2 and had more suppressed respiratory movement and became more cyanotic after the APGAR first evaluation, and animal 2 (FIX2) presented APGAR score 3. After 5 min post partum, APGAR score of FIX1 and FIX2 were 2 and 6 respectively. Atropine (0.05mg/kg; IV) was administered in both animals in attempt to revert bradycardia. Also, intranasal oxygen (5L/min), aminophylline (6mg/kg, IV), DEX (0.05mg/kg), bromhexine hydrochloride (0.5mg/kg; SC), and sildenafil (25mg, oral) were administered 10 minutes after delivery, in order to improve pulmonary function. Although treatments, FIX1 died about one hour, and FIX2 died about 6 hours after delivery. Clinically, FIX1 umbilical cord was increased in size, presenting approx. 85mm of diameter. FIX1 necropsy findings include hemorrhagic umbilical arteries increased in size and collapsed nonfunctional lung, fat degeneration of the liver, degenerated kidneys, pericardium excess fat, and hematic cyst at the heart valves. FIX2 had an improvement of physiological parameters after administration of medicines; nevertheless, it was feed by nasogastric tube. Although FIX2 had smaller umbilical cord (about 35mm) it was still augmented. FIX2 necropsy findings included internal bleeding in umbilical cord region next to abdominal ventral wall only. We conclude that neonatal care protocol could improve pulmonary function in some newborns presenting certain degree of lung maturation in cloned calves.



A244 Cloning, transgenesis and stem cells

### **Generation of bovine induced pluripotent stem cells (iPS) from Rho 0 mesenchymal bovine cells using human transcription factors**

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**Keywords:** cells, iPS, Rho 0.

This study aimed to use bovine Rho 0 mesenchymal cells obtained through treatment with Ethidium Bromide (EtBr) to the conditions necessary to produce induced pluripotent stem cells (iPS) and to evaluate them regarding their mitochondrial DNA (mtDNA) content and gene expression of Tfam, Bax and Bcl-2. Rho 0 cells used in this experiment were produced in previous experiments using defined EtBr concentration (100ng/mL) and culture duration established based on King and Attardi (King 1996, Method in Enzymology, 264, 304-313). Cells were distributed in four experimental groups: control Rho 0 (Rho 0c); transduced Rho 0 (Rho 0t) both kept in culture in the presence of EtBr; control MSC (MSCc) and transduced MSC (MSCt), kept in culture in the absence of EtBr. Groups were then submitted to the process of transduction for viral insertion of the human vector STEMCCA (hSTEMCCA) containing transcriptional factors Oct4, Sox2, Klf4 e c-Myc sequences. These cells were cultured for 21 days, in ideal conditions at 38.5°C, 5% CO<sub>2</sub> atmosphere and maximum humidity. Until the fifth day after transduction cells were cultured in supplemented IMDM medium (10% fetal calf serum; 1% Antibiotics), after the fifth day cells were cultured in supplemented DMEN/F12 KnockOut medium (20% KnockOut Serum Replacer; 1% Antibiotics; 1% Non-essential Aminoacids; 1% Glutamine; 0,007% β-mercaptoethanol; 0,01% bFGF). After a 21-days period, cells were collected and evaluated considering their mtDNA content and relative quantification of genes Tfam, Bax, Bcl-2 and the ratio Bax/Bcl-2. Regarding mtDNA copy number, there was no statistical difference after transduction between MSC and Rho 0 groups (P=0,10 and P=0,57, respectively) and also no interaction among experimental groups was found. It may be observed that there was no effect of the transduction process and treatment with EtBr during the process. Concerning relative quantification, groups didn't present difference for Tfam after transduction (P=0,54 and P=0,19, respectively for groups MSC and Rho 0). Groups also didn't present difference for Bax expression (P=0,87 and p=0,18, respectively for groups MSC and Rho 0). However, after transduction, groups Rho 0c and Rho 0t presented increase of Bcl-2 expression (P=0,02). After transduction, none of the groups presented expression alterations for Bax/Bcl-2 ratio. Despite there is interaction among studied variables, which is capable of influencing Bcl-2 expression for Rho 0c and Rho 0t groups and presenting tendencies, it can be concluded that the hSTEMCCA vector system associated or not to a mtDNA copy number reduction is not capable to induce bovine mesenchymal cells to a pluripotent state, once cells didn't succeed in forming colonies and didn't present morphological modifications.



A245 Cloning, transgenesis and stem cells

### **Allele-specific expression of the MAO-A gene and the X chromosome inactivation in bovine embryos produced by nuclear transfer**

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**Keywords:** embryoblast, RT-PCR-RFLP, trophoblast.

The inactivation of one of the X chromosomes (XCI) in females equalizes gene expression between the sexes (Lyon 1961, *Nature*, 190, 372-373). The XCI in cloned embryos can show some alterations, as such as failures in the pattern of imprinting in the placenta (Yang *et al.* 2007, *Nat Genet*, 39, 295-302). Therefore, it is suggested that epigenetic reprogramming occurs incompletely at most clone embryos, resulting in an aberrant gene expression and abnormal embryonic development (Morgan *et al.* 2005, *Hum Mol Genet*, 14, 47-58). Thus, the aim of this study was to characterize the process of XCI in trophoblast (TP) and the inner cell mass (ICM) of bovine embryos produced by nuclear transfer (NT) through the characterization of allele-specific expression of the MAO-A gene (located on the X chromosome and subject to inactivation). We used fibroblasts from a skin biopsy of a female adult Nellore, previously genotyped for MAO-A gene and presenting AG genotype. The cumulus oocyte complexes from slaughterhouse ovaries were evaluated for the presence of the first polar body (PB) after 20 hours of maturation. Oocytes showing PB were subjected to the process of nuclear transfer, which were held individually by holding pipette and a portion of the cytoplasm adjacent to the PB was removed. A somatic cell was then placed in the perivitelline space and the structures were subjected to electrofusion (ECM 200, BTX<sup>®</sup>) with two pulses of 2.1kVA with 50µs of duration. After 30 minutes, oocytes were activated using ionomicina/6-DMAP for 4-5 hours followed by *in vitro* culture in SOFaaci medium for eight days in an incubator at 39 ° C and 5% CO<sub>2</sub> in air. It was produced a total of 25 embryos. In D8, Bx embryos were taken to the micromanipulator to separate ICM and TP. Total RNA was extracted from biopsies of TF and ICM individually using the Arcturus<sup>®</sup> Peak Pure RNA Isolation Kit (Life Technologies<sup>®</sup>). For cDNA synthesis was used Oligo dT primers (Invitrogen<sup>®</sup>) and SuperScript III reverse transcriptase (Invitrogen<sup>®</sup>). It was used RT-PCR-RFLP technique to detect and characterize the allele-specific expression of the MAO-A gene and 13U for digestion of restriction enzyme RsaI (Promega<sup>®</sup>). Despite the possibility of the presence of trophoblast cells in biopsies of MCI, biallelic expression of the MAO-A gene in clone embryos (n = 25) were 32% (n = 8) in ICM and 32% (n = 8) in TP, with a prevalence of detection of G allele compared to A allele, in MCI (96% vs. 36%) and TP (100% vs 36%). We suggest that at this stage of development, the XCI is not random in ICM and TP embryo clones of cattle, or that there is an alteration induced by TN, causing an abnormal expression or some deviation in the process of XCI favoring the inactivation specific alleles, as the G allele.

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A246 Cloning, transgenesis and stem cells

### **In vitro production of bovine tetraploid embryos after blastomeres fusion at two cell stage**

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**Keywords:** blastocysts, fusion, Tetraploid.

With the purpose of obtaining descendants from genetically superior animals, studies with in vitro embryo production were carried out and last generation biotechnologies have been developed, as NT. However, several anomalies have been related to animals produced by this technique. In bovines, peri-implantation losses are estimated at 50%. These losses are frequently associated with functional deficiencies that occur in early placentation. The concept of embryonic complementation consists in grouping diploid/tetraploid (2n/4n) cells with a non-random distribution of these cells. Tetraploid cells will contribute to the formation of extra-embryonic tissues while 2n cells will form the ICM. To obtain 4n embryos, 2-cell embryos can undergo blastomeres fusion or electrofusion. The aim of this study was to evaluate the use of polyethylene glycol (PEG) to fuse blastomeres of 2-cell stage embryo and subsequent embryo development toward blastocyst stage. The COCs were aspirated from slaughterhouse ovaries, selected and transferred to 100  $\mu$ L droplets of TCM 199 and in vitro matured for a period of 22-24 hours. IVF was performed with semen from a single bull with proven fertility. SOF was the medium used for embryo culture. Embryos were maintained at 38.5 ° C and 5% CO<sub>2</sub> throughout the culture. To perform cell fusion, 30-35 hours post fertilization (hpf) embryos with 2 blastomeres were selected and then inserted for 1 minute in medium containing 40% polyethylene glycol (PEG). The embryonic development was evaluated in four steps: (1) cleavage at 30-35 hpf, (2) fusion rate 3-4 hours after PEG treatment, (3) cleavage at D4 post fertilization, (4) blastocyst rate at D8 post fertilization. The experiment consisted of the following groups: G1 – non-fused 2-cell stage embryos, (G2)- fused 2-cell stage embryos after PEG treatment and G3 – non-cleaved embryos or embryos with more than 2 cells at the moment of fusion with PEG. Data were analyzed by ANOVA and the 't' test of Student. We found: (1) 66.03% of the embryos cleaved at 30-35 hpf, (2) 90.9% of embryos lost their shaft between the blastomeres, (3) at D4 the cleavage rate was 70% for G1, 81.81% for G2 and 74.32% for G3, (4) at D8 the blastocyst production rate was 50%, 63.64% and 49.61% for G1, G2 and G3 respectively. We can conclude that PEG can be used to fuse blastomeres and produce tetraploid embryos. Further studies will be carried out to analyze the embryos karyotype.



A247 Supporting biotechnologies

### Uterine blood flow evaluation after artificial insemination on different uterine sites in mares

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**Keywords:** blood flow, mare, uterus.

The intensity of inflammatory response depends on concentration and volume of inseminate. In addition, insemination with very small volumes may result in less effective mechanical drainage, while highly concentrate semen may be more irritating because of more contacts between spermatozoa and endometrium, resulting in intense inflammatory response (Kotilainen *et al.*, 1994, *Theriogenology*, 41, 629-36). Doppler mode ultrasonography, when association with B mode, brings information about vascular architecture in real time and hemodynamic aspects of vessels in different organs (Carvalho *et al.*, 2008, *Ciência Rural*, 38, 872-79). The aim of this study was to evaluate both uterine horns and uterine arteries in mares inseminated into the uterine body or into the tip of the uterine horn by Spectral Doppler mode ultrasonography. Sixteen mares were inseminated with cooled jack semen until six hours after ovulation, into the uterine body (volume of 20mL and 500 million spermatozoa) or into the tip of the uterine horn, ipsilateral to the dominant preovulatory follicle (4mL and 100 million spermatozoa), with eight mares in each group. RI (resistance index) was determined for a mesometrial artery in each uterine horn and for both uterine arteries, using the mean of three values for data analyses. Analyses of variance and Duncan test were used for analyses of data and means, respectively. There were no difference between the group inseminated into the uterine body or into the tip of the uterus when compared the values of mesometrial and uterine arteries RI ( $P > 0.05$ ). The following data was obtained: group inseminated into the uterine body (right =  $0,78 \pm 0,08$  and left =  $0,77 \pm 0,075$  mesometrial RI; right =  $0,88 \pm 0,06$  and left =  $0,87 \pm 0,09$  uterine arteries); group inseminated into the tip of the right uterine horn (right =  $0,76 \pm 0,07$  and left =  $0,78 \pm 0,08$  mesometrial RI; right =  $0,89 \pm 0,05$  and left =  $0,88 \pm 0,05$  uterine arteries); and group inseminated into the tip of the left uterine horn (right =  $0,80 \pm 0,08$  and left =  $0,81 \pm 0,07$  mesometrial RI; right =  $0,88 \pm 0,03$  and left =  $0,89 \pm 0,06$  uterine arteries). In conclusion, uterine vascular perfusion variation, owing to inflammatory response after exposure to different volumes and concentrations of inseminate doses, was not detected using only RI evaluation of mesometrial and uterine arteries.



A248 Supporting Biotechnologies

## Effects of glycerol and ethylene glycol association on ovine sperm cryopreservation

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**Keywords:** cryoprotectant, semen extender, spermatozoa.

Glycerol is frequently used as cryoprotectant to freeze ruminants' semen, however it has toxic effects to the sperm. Alternatively, ethylene glycol has been used due to its cryoprotectant characteristics, lower molecular weight and easiness of crossing the plasma membrane. This study aimed to compare the effects of the combination of ethylene glycol and glycerol as cryoprotectants for freezing ram semen through the post-thaw assessment of total (MT) and progressive motility (MP), vigour (V), and through the evaluation of the integrity of plasmatic, acrosomal and mitochondrial membranes using the fluorescent probes propidium iodide, FITC-PSA and JC1 respectively. Ejaculates from 17 animals collected with artificial vagina and with values equal to or greater than 70% MV and MP were used. Semen cooling was performed in two steps, into polystyrene boxes. Initially semen was diluted in fraction A (FA: TRIS-egg yolk), and incubated for 2 hours when temperature reached 5°C, followed by the addition of fraction B (FA + cryoprotectant) (1:1) containing 14% glycerol, 6% ethylene glycol (control groups) or glycerol 7% + ethylene glycol + 3% (experimental group), with additional incubation for 30 minutes at 5°C. The semen was packaged in 0.25 ml straws and placed 6 cm above the liquid nitrogen (N2L) level, for 15 minutes and then dipped in N2L. The straws were thawed in water at 37°C. The physical parameters were analyzed subjectively. The evaluations of membranes were made using fluorescent probes by counting 200 cells of each sample, with the aid of epifluorescence microscopy. Data were subjected to analysis of variance and means were compared by t test at 5% significance. The results do not show significant differences in physical parameters between samples frozen with glycerol alone (MT = 48.52 ± 18.27; MP = 34.11 ± 8.52 and V = 3.47 ± 0.87) and ethylene glycol (MT = 37.05 ± 18.54; MP = 27.94 ± 13.58 and V = 3.12 ± 0.78), or with the combination of glycerol + ethylene glycol (MT = 47.35 ± 15.52, MP = 36.17 ± 10.68 and V = 3.76 ± 0.56). The evaluation of sperm membranes by fluorescent probes propidium iodide, FITC-PSA and JC1 showed no differences between samples frozen in the presence of glycerol (63.39% ± 46.46, 65.83 ± 35.00% ; 28.21 ± 49.17%), ethylene glycol (54 ± 21% 47.54, 63.83 ± 44.23, 50.51 ± 39.59%) or combination of glycerol and ethylene glycol (57, 09% ± 38.83, 33.14 ± 41.64%; 52, 38% ± 23.16) respectively. Since the different treatments showed similar results for all parameters evaluated, the combination of cryoprotectants glycerol and ethylene glycol can be used in the cryopreservation of ovine semen, but it is important to evaluate other semen parameters such as the rate of pregnancy to obtain information about the possible benefits of cryoprotectants association.



A249 Supporting Biotechnologies

### **Omega 6 and 3 polyunsaturated fatty acids association during *in vitro* production did not alter the cryotolerance and membrane lipids profile of bovine embryos**

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**Keywords:** cryotolerance, embryo, mass spectrometry.

Studies report positive effects of polyunsaturated fatty acids (PUFAs) supplementation, such as conjugated linoleic acid (CLA, omega 6) and docosahexaenoic acid (DHA, omega 3), during *in vitro* production of bovine embryos, on its cryotolerance. However, the effect of the association of omega 6 (n-6) and 3 (n-3) PUFAs families during embryo IVP, has not been evaluated. This study was conducted in order to evaluate the effects of supplementation of CLA and DHA association, during IVM and IVC on cryotolerance and membrane lipid profile by matrix-assisted laser desorption/ionization - mass spectrometry (MALDI-MS), of IVP bovine embryos. COCs (n=491) were IVM for 22 h, and after IVF, zygotes were IVC in SOFaa medium (5 mg/mL BSA + 2.5% FCS, 5% CO<sub>2</sub> in air) for 7 days. The experimental groups were: Control (C) and 100 μM CLA + 100 μM DHA (CLA+DHA). Blastocysts were vitrified (Ingamed<sup>®</sup>, Maringá-PR, Brazil) and after 3 h of thawing, embryo survival rates were measured. Viable embryos were transferred to microtubes containing 200 μL of methanol HPLC 50% in aqueous solution, stored at -20 °C and immediately transported for MS analysis. Each embryo was deposited at the center of the spot's plate. Before MALDI-MS analysis, 1 μL of matrix (1.0 mol/12.5 dihydroxybenzoic acid (DHB) in methanol) was deposited on each spot and dried at room temperature until its complete crystallization. Spectra were acquired in the mass range of *m/z* 700-1200, in the positive ion and reflectron modes using an Autoflex III (Bruker Daltonics, USA) mass spectrometer. After excluding isotope peaks, the most intense ions of each spectrum were considered as starting point for determining the *m/z* ratios corresponding to membrane lipids. Only *m/z* clearly distinguished from noise were included in the partial least squares discriminant analysis (PLS-DA). The re-expansion rates were evaluated by qui-square test. These rates did not differ ( $P > 0.05$ ) between groups were assessed and 63.5% (C) and 62.1% (CLA + DHA). The association of PUFA n-6 and n-3 did not influence embryonic cryotolerance success rates. In agreement with the cryopreservation results, no variations on membrane lipid profile were observed in CLA + DHA group, compared with C group embryos. In this case, there was no separation of the groups in PLS-DA, indicating that sphingomyelin (SM) and phosphatidylcholine (PC) profiles were not affected by treatment. In conclusion, the supplementation with the association of CLA + DHA during IVM and IVC had no effect on cryotolerance and membrane lipid profile of IVP bovine embryos.



A250 Supporting Biotechnologies

### **Biomodulation of MAPK expression by low level laser on oocyte in vitro maturation in cattle**

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**Keywords:** Low-level laser, in vitro maturation, cattle.

Low-level laser irradiation (LLLI) is an alternative for the biomodulation of oocytes in order to improve blastocyst formation after fertilization. Two isoforms of MAPK, known as ERK1 / 2, are activated near the VG breakdown in bovine oocytes been essential for the transmission of maturation and cumulus cells expansion signals, both *in vitro* and *in vivo*. The objective of this study was to evaluate the effect of LLLI ( $\lambda$  633nm HeNe - fluency  $1\text{J}/\text{cm}^2$  - 16:30 min) at the beginning of oocyte IVM in the MAPK (ERK1 / 2) expression when compared to non-irradiated oocytes (control). Control and irradiated samples (at least 3 replicates per group) were evaluated by the amount of Total MAPK and phosphorylated MAPK (MAPKP) after 30 min, 8 hours, 16 hours and 24 hours of IVM. The quantification of MAPK and MAPKP was performed by Western blot from 10 oocytes using a digestion buffer (1% NP40, 135 mM NaCl, 20 mM Tris pH 8, 10% glycerol). Protein integrity was analyzed by the Bradford method. Protein bands were quantified by densitometry and their densities calculated in arbitrary units. This value was submitted to 2WAY ANOVA with Bonferroni post test (Prism 5 GraphPad Inc). At the end of IVM oocytes were also evaluated for extrusion of the first polar body by optical microscopy and subjected to in vitro fertilization. There was an interaction between time and treatment for total MAPK ( $p < 0.001$ ). There was no difference between irradiated and control group for total MAPK, except for 8 hours group in which the irradiated oocytes presented ten times higher amount of protein than the control. Despite the increase in total MAPK, there was no difference between the groups regarding MAPKP. This corroborates with the data related to the MAPK total / MAPKP ratio, which resulted in higher values 8 hours after the beginning of maturation. Despite this increase, the number of oocytes with nuclear maturation (extrusion of the first polar body) remained the same between groups, as cleavage and blastocyst rates after IVF. In fact it is expected an increase in levels of MAPK between 8 and 14 hours. However, the increase of MAPKP in 8 hours did not follow the pattern total MAPK. We concluded that the irradiation LLLT was able to induce increased expression of the total MAPK in in vitro matured bovine oocytes. More studies are needed to induce physiological changes induced by LLLT that can be used as an alternative aimed at better support for future events of fertilization.

**Acknowledgments:** FAPESP and UFABC.



A251 Supporting Biotechnologies

### **Synchronization of the follicular wave and blood flow of the dominant follicle in crossbred heifers treated with estradiol benzoate and cypionate**

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**Keywords:** colour Doppler, follicular dynamics, FTET.

The study was designed to compare the synchronization of the follicular wave and the blood flow of dominant follicle after estradiol cypionate (EC) or benzoate (EB) treatment. Seventy-five crossbred heifers (HxZ) were distributed in 2 experiments (Exp.) and the same hormone protocol was given: Day zero (D0), insertion of the P<sub>4</sub> implant (1,0g Primer®, Tecnopec); D8, removal of the implant and injection of the sodium Cloprostenol (0.5 mg, Sincrosin®, Vallé). For Exp.1, 45 heifers were distributed in three groups (G), according to estradiol treatment on D0: G1a (n=15) 1ml of IM salina; G2a (n=15) 0.5 mg of IM EC (Von Franken®); and G3 (n=15) 2.0 mg of IM EB (Ric-BE®, Tecnopec). The diameter of the largest follicle – present at the ovary before treatment - was tracked on during days: 0, 2, 3, 4 and 5. The emergence of the new follicular wave – first observation of the dominant follicle at ≥4 mm – was also recorded. For Exp.2, the follicular wave was synchronized with 2.0 mg of EB (D0, n=30) and the ovulation was induced on D9 with one of the treatments: Gb1, 1 ml of IM saline (n=10); G2b, 0.5 mg of IM EC (n=10); and G3b, 1.0 mg of IM EB (n=10). From D8 to D10, the diameter and blood flow of the dominant follicle was monitored with an ultrasound device, equipped with color Doppler (7.5 MHz, M5, DPS-Equipamentos Médicos, São Paulo). In the cross-section of the follicle with the highest blood flow, score of vascularization was designated, according to percentage of the follicular wall occupied by color signals: 1 (>75%), 2 (>50 and ≤75%), 3 (>25 and ≤50%) and 4 (≤25%). Data for follicle diameter were checked for normality and analyzed with ANOVA, for the main effects of the group, day and interaction. The averages of the groups were compared by Tukey test to 5% (5-10% approach). Data for blood flow scores were analyzed in Kruskal-Wallis procedures. In Exp.1, the regression of the largest follicle was more effective (P<0.07) between 0 and 2 (-1.0±0.4 vs -0.2±0.2mm) in EB group. The effect of day was not significant (P>0.05) in control group (saline) group. The emergence of the new follicular wave was observed 3,5 days after the beginning of the protocol in CE and about 1 day later in the EB group (4.3d, P<0.0013). In Exp.2, the diameter of the dominant follicle did not differ (P>0.1) among the groups. The blood flow of the dominant follicle increased (P<0.0006) 24h (D9 to D10) after treatment in EB, which has not been observed in EC and saline groups. The ovulation rate did not differ (P>0.05) between groups (80, 70 and 60%, respectively for BE, CE and saline, respectively). It is concluded that the two estradiol treatments were efficient to synchronize a new follicular wave, however, the dose of BE may have contributed to the delay in follicular emergence. The increase in blood flow of the dominant follicle, 24h after EB treatment, might indicate close relation of the LH surge, as blood flow increases with the preovulatory peak of the LH surge (Acosta et al., 2003, Reproduction, 125, 759-67).

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A252 Supporting Biotechnologies

### Ultrasound features of the corpus luteum 21 days after estrous and pregnancy diagnosis of bovine recipients: preliminary results

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**Keywords:** colour doppler, early diagnosis, FTET.

The study was designed to relate ultrasound (US) features – B mode and color Doppler (CD) mode - of the CL, 14 days after embryo transfer, and pregnancy diagnosis of the embryo recipients. Sixty-one (n = 61) recipients, synchronized (estrous = D0) and timed embryo transferred (TET, around D7) of frozen embryos (Ethylene glycol 1.5 mol) from South Africa (Embryo Plus, Brits). Fourteen days later (D21), the CL was classified according to its size and blood flow: score 1 (Large; High blood flow in most of the gland), 2 (Medium; Regular or Concentrated blood flow in certain areas of the gland) and 3 (Small; Poor or low blood flow). All procedures were performed by the same ultrasound operator. Approximately 400 frames in B mode and 100 in color-flow Doppler, containing entire cross-sections of the CL, were firstly stored in *avi* format on the ultrasound device (M5, DPS medical equipment, Sao Paulo), and further transferred to an external HD (Samsung 500 GB). The area of the CL at largest diameter and averaged area of the CL with colored pixels - indicative of blood flow – from three regions (central and opposite sides of the CL) were measured with ImageJ software (Image Processing and Analysis in Java). The predictive diagnosis (DG21) was based on CL at the same ovary of D7 and blood flow score of 1 or 2. The CL and blood flow areas were compared to US scores for size (B mode) and blood flow (DP). The PROC GLM procedure of SAS statistical software (version 9.0, SAS Institute Inc., Cary, NC, USA.) and the Duncan test were used to access the differences among means. Pregnancy diagnosis was performed on D35 (DG35) and the PROC FREQ procedure used to test the agreement of DG21 and DG35. The predicted pregnancy rate (DG21) was 57.4% (34/61) and did not differ ( $P > 0.05$ ) from the ultimate diagnosis (DG35 = 50.8%, 31/61). Non-pregnant animals were accurately detected on DG21 (100% predictive value for negative) and its predictive value for positive was also high (88.6%). These results were similar to other early diagnosis of pregnancy based on functionality of the CL (Siqueira et al., In press). Thus, corpus luteum of pregnant animals had shown larger total ( $305.8 \pm 59.4$  vs.  $111.6 \pm 55.3$  mm<sup>2</sup>,  $P < 0.0001$ ) and blood flow area ( $64.5 \pm 22.3$  vs.  $5.2 \pm 7.2$  mm<sup>2</sup>,  $P < 0.0001$ ), when compared to non-pregnant animals. Noticing that the CL area was 3x larger and the difference in blood flow area was 12x higher in pregnant compared to non-pregnant recipients, it is possible to conclude that the diagnosis of pregnancy based on the size and blood flow of the CL is feasible as early as 14 days after embryo transfer.

**Acknowledgments:** Biotran e FAPEMIG (financial support, project number APQ-1454-12).



A253 Supporting Biotechnologies

### **Ultrasonography evaluation of fetal sexing in mares at 60 days of gestation after embryo transfer under farm condition**

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**Keywords:** mare, sexing, ultrasound.

Embryo transfer technology in horses was developed to increase their genetic value. Recently, the improvement of ultrasound equipment has allowed more accurate identifications of fetal sex. The objective of this study was to evaluate in a program of embryo transfer in horses the efficiency of fetal diagnosis at 60 days of pregnancy. Twenty-eight mares from La Vanguardia haras, located in San Andrés de Giles-Argentina, were used. The embryos were produced by artificial insemination and collected seven days after ovulation. They were soon transferred to the twenty-eight recipients. Pregnancies were confirmed seven days post-transfer by ultrasound by visualization of the embryonic vesicle. Ultrasonographic evaluation was done using an ultrasound (Well D Medical Electronics®, Shenzhen, China) coupled to a transrectal probe from 5.5 to 7.5 MHz. The position of the genital tubercle was evaluated at 60 days of gestation. When the genital tubercle was located caudal to the umbilical cord the fetus was considered as male, and if it was near to the fetus tail it was considered as female. At 120 days of gestation fetal sex was confirmed according to Renaudin et al., 2000 (J Reprod Fertil Suppl., 56, 651). The accuracy of the gender diagnosis at 60 days was evaluated at 120 days, the correct sex of male and female were compared by Qui-square test. The accuracy of pregnancy diagnosis at day 60 after confirmation day 120 of gestation was 87.71% (24/28). The difference between male and female fetuses with incorrect diagnoses was significant ( $P < 0.05$ ), 20% (3/15) for female and 7.69% (1/13) for male. These results are similar to those found by Merkt et al., 1999 (Journal Medicine Vet Sci, 19, 90) as they reported that female fetuses are more difficult to diagnose than male fetuses. The accuracy of sexing at 60 days of embryo transfer has not reached 100%.

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A254 Supporting Biotechnologies

### **Milk proteins associate to goat binder-of-sperm (BSP) homologs preventing the binding to epididymal sperm membrane in vitro**

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**Keywords:** binder-of-sperm, goat, milk proteins.

Previous studies show that Binder-of-Sperm (BSP) proteins from bovine seminal plasma (SP) associate with milk proteins (Bergeron et al. 2006, 2007a, 2007b, Lusignan et al. 2011). Although studies confirm the importance of BSP proteins in the bovine, interactions of BSP homologs from other species and extender components have not been evaluated. Thus, the present study was conducted to evaluate if milk protein associate to goat BSP proteins *in vitro*, minimizing the binding to epididymal sperm membranes. Spermatozoa were recovered from the cauda epididymis from bucks by retrograde flushing and incubated with either skim milk (Pellicer-Rubio et al 1997) or citrate-glucose medium (2.37 g sodium citrate, 0.8 g glucose, 100 ml distilled water) at 37°C. Following incubation, an appropriate amount of crude goat SP was added. In parallel, ejaculated and epididymal sperm were subjected to the same approach as control. Sperm membrane protein extraction was performed according to the method described by van Tilburg *et al.* (2013). Protein samples from each preparation were separated by SDS-PAGE and the presence of goat BSP proteins was assessed by immunoblotting using a polyclonal antibodies raised directly against goat BSP protein homologs (GSP-14, GSP-15, GSP-20 and GSP-22). Immunoreactivity of goat BSP proteins was detected in the ejaculated sperm and citrate-glucose medium fractions, indicating that goat BSP proteins interact with sperm membrane. In contrast, when goat SP proteins were added to skim milk medium plus caudal epididymal sperm, the polyclonal antibodies could recognize weakly those goat BSP proteins, suggesting that these proteins associate to milk proteins, minimizing the binding to sperm membrane. The decrease of goat BSP proteins suggests that skim milk extender plays a significant role in protecting goat sperm during storage. Certainly, the protein-protein interaction prevents an extensive loss of lipids from sperm membranes which may be deleterious to sperm storage. In summary, our study showed that BSP proteins present in goat SP have affinity for the milk proteins. The results obtained in the present study confirm the hypothesis that skim milk components sequester BSP proteins, preventing the detrimental effects of these proteins on the sperm membrane. These findings are of considerable interest in view of the mechanisms of sperm protection by extender constituents.

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A255 Supporting Biotechnologies

## **Software based on artificial neural networks for embryo morphologic evaluation**

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**Keywords:** classification, embryology, software.

The embryonary morphologic classification – of great importance for various researches, from basic to applied on assisted reproduction – is still based on subjectivity and have no precise, consistent and trustful method (Farin et al., 1995, Theriogenology, 44, 339-49). The main goal of this work was to develop a protocol for information extraction (based on digital bidimensional images) and a software, for the morphological classification of mammals embryos in a similar way that an embryologist would do, having Artificial Neural Networks (ANN) and Digital Image Processing as base. An image database was developed with 98 samples of mice blastocysts (between initial and expanded stages) obtained from superovulation of Swiss Webster mice. Using these images, a protocol of information extraction was developed, in a way that 12 variables were obtained. Among these variables, for example, there was the development stage, days-post- mating, embryo area and its circularity. The variables worked as an input for the ANN classification in four different degrees of quality (excellent, good, fair or bad). As base of training for the ANN, the same images were classified in a conventional way (visual evaluation from an experienced embryologist). From all images on the database, 80% were used for the learning process and 20% for the proof of efficiency (test data) of the ANN. The comparison of results (between the output of the ANN and the embryologist analysis) verified that 75% of the classifications were successful. To verify the consistency (repeatability) of the embryologist analysis was settled a blind test, on which the test images were categorized by the embryologist again (without the knowledge of the original classification). Between the first and second evaluation, there was variation of classification on 7 images. When both classifications made by the embryologist are considered as correct, that is, as possible variations of morphological quality, the success rate of the software increases to 95% (only one misclassification). For the final version of the software was developed a graphical user interface, in a way that it could be used by technicians and students of the field, only demanding a minimum knowledge of embryology. The process presented a high potential of applicability, specially having in mind the possibility of expansion for other mammal species with more commercial interest (as human and bovine). The whole process, or more precisely, from the mining of variables from the embryo image to the utilization of the final software for analysis, was framed as patentable and its deposit made at the Brazilian National Institute of Industrial Property (INPI). In conclusion, the software prototype, obtained from mice embryo images, presented high analysis objectivity, consistency and a higher precision then the visual embryologist analysis.

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A256 Supporting Biotechnologies

### Preimplantion genetic diagnosis using whole genome amplification of equine embryo biopsy

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**Keywords:** biopsy, equine, sexing.

Although Brazil is the world's leader in equine embryo transfer, the techniques of micromanipulation and preimplantation genetic diagnosis are still underdeveloped in the country. Recent studies showed that embryo biopsy can be performed without affecting the conception rate and can improve the results on vitrification of expanded blastocyst. The aim of this work was to develop the techniques for micromanipulation and molecular biology to identify the sex of equine embryos. The biopsy was performed by microaspiration using two micropipettes (Holding and Biopsy) coupled to mechanical micromanipulators (Narishige), enabling the drilling of embryonic capsule without using Piezo Drill, acid or laser. The standardization of PCR was performed with genomic DNA (gDNA) samples extracted from horse and mare blood, quantified (NanoDrop), adjusted to the concentration of 50ng/μL and subjected to serial dilutions (5ng/μL, 0.5ng/μL, 50pg/μL and 5pg/μL), enabling the assessment of sensitivity in two primers: Y-specific (SRY) and endogenous control (S4B). The result was visualized by electrophoresis in Agarose gel stained with ethidium bromide. Although the primer S4B showed great sensitivity, the primer SRY showed no band in male samples with concentrations below 50pg/μL, revealing that it will not be possible to identify the sex from embryo biopsy (10-20 cells). Thus, we used the Whole Genome Amplification methodology in order to increase the gDNA amount from aliquots of 10-20 fibroblastic cells of horse and mare obtained from *in vitro* culture. Subsequently, four thawed equine embryos were submitted to repeated biopsies of ~20 cells, enabling the standardization of WGA and PCR methodology with primers S4B (145bp) and SRY (182bp), totaling approximately 12 hours, from biopsy to electrophoresis. The result with fibroblastic cells allowed the visualization of the two bands on male sample and only one (S4B) in female samples. The result of embryo sexing revealed two male e two females, suggesting that the methodology developed in this work can be used for equine embryo sexing. Two equine embryos were biopsied immediately after collection and kept in maintenance medium (Holding) at 37°C for 12 hours. Evaluation of embryonic morphology was carried out at 3, 6 and 12 hours after biopsy, allowing to observe the reorganization of embryonic structures as well as the blastocoel reconstitution, which indicates the embryos remained viable after biopsy. The next stage of the work will be to conduct field experiments that will assess the conception rate of biopsied equine embryos and fresh transferred, as well as biopsied and vitrified embryos.



A257 Supporting Biotechnologies

### **Preliminary investigation on effect of fetal bovine serum in the expression of genes involved in via cGMP during in vitro maturation bovine oocyte**

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**Keywords:** cGMP, FCS, maturation.

The sensitivity of IVP embryos to cryopreservation is often associated with lipid accumulation in the cytoplasm. Among the possible causes of this event, the presence of fetal calf serum (FCS) in the culture medium (Barceló-Fimbres et al., 2007, Mol Reprod Dev, 74, 1395-405) or other disorders in energy metabolism have been identified (Sanchez et al., 2006, Reprod Fert Dev, 18, 585-96). In adipocytes of primates, including humans, lipolysis-related hormones, such as hormone-sensitive lipase (HSL), are activated when phosphorylated by both cAMP- (PKA) and cGMP-dependent protein kinase (Lafontan et al., 2008, Trends Endocrin Metab, 19, 130-7). Thus, intracellular levels of cAMP and cGMP are involved in the regulation of the rate of lipolysis in adipocytes; when they are high they cause lipolysis while when reduced promote lipogenesis (Hass et al. 2009, Science, 2, 78-89). Both nucleotides are also present in bovine oocytes, together with their synthesis (GC and AC) and degradation enzymes (PDE3 and PDE5, Schwarz, 2011, PhD Thesis, FZEA-USP, 106f). The aim of this study was to evaluate the influence of fetal calf serum (FCS) on the transcripts of some components cGMP pathway. For this, COCs were cultured for 24h in maturation medium supplemented with different proportions of FCS (2% to 10%). The control group was matured in medium only with 0.4% BSA. The mRNA of pools of 20 denuded oocytes and their respective cumulus cells from each group was extracted with Trizol and converted using the High Capacity cDNA Reverse Transcription Kit. The relative amount of genes linked to the control of cGMP levels (GUCY1B3 and PDE5A) or enzymes activated by it (PKG1 and PKG2) and the gene for beta oxidation of long-chain fatty acids (CPT1B) was determined by Real Time PCR using SYBR Green. The data relating to five replicates were analyzed with PCR program LinReg. Based on the analysis performed with the program Genorm, the geometric mean of the expression of genes GAPDH and PPIA (Lonergan et al., 2003, Reprod Biomed Online, 7, 657-63) were used as endogenous controls. Statistical analysis was performed by ANOVA followed by Tukey post-hoc at a significance level of 5%. All analyzed genes were expressed in both cellular compartments. GUCY1B3 reduced expression in oocytes, whereas in cumulus cells PDE5A increased the expression and CPT1B decreased ( $P < 0.05$ ) when cultivating COCs in the presence of FCS in comparison with the control group. In conclusion, FCS affected the relative expression of genes of the cGMP pathway that may be related to lipid metabolism in COCs. Studies are being conducted to confirm the effect of FCS on lipid accumulation and to determine the correlation with the accumulation of cGMP pathway in bovine COCs matured in vitro.



A258 Supporting Biotechnologies

## RNA sequencing (RNA-Seq) of in vitro and in vivo bovine embryos

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**Keywords:** bovine, embryo, RNA-Seq.

Several technologies have been explored to highlight differences among embryos towards identification of those most likely to establish successful pregnancy. In this sense, transcriptome sequencing (RNA-Seq) is a powerful tool that can provide valuable information about the global gene expression of embryos. This information may help in understanding important metabolic pathways and identify patterns of in vivo development necessary for further improvement of IVP. The aim of this study was to evaluate the transcriptomic profile of bovine embryos produced in vitro from “fast” (4 cells at 40 hpi and blastocysts derived from this group) and “slow” (2 cells at 40hpi and blastocysts derived from this group) developmental groups as well as in vivo embryos. For this purpose, in vitro embryos were produced by conventional methods and cultured individually until the moment of analysis (in triplicate with 10 embryos per group). Blastocysts were produced in vivo and collected by conventional methods (in duplicate with 10 embryos per group). Total RNA was extracted, quantified and analyzed for integrity with an Agilent 2100 Bioanalyzer. The recovery of total RNA ranged from 131 to 521 pg per cleaved embryo and from 439 to 1319 pg per blastocyst. All RNA obtained was used for cDNA synthesis and subsequent amplification using the Nugen Ovation V2 kit. After amplification, the product obtained was quantified and evaluated. Between 2.23 to 5.2 µg of amplified cDNA was obtained from samples of cleaved embryos and between 2.38 and 4.76 µg from blastocysts. Samples with cDNA fragments between 600 and 3000 bp were considered suitable for the construction of sequencing libraries. The samples were then sonicated to fragments of approximately 300 bp. From these samples, 1 µg was used for construction of the libraries with the Illumina TruSeq Library system. Sequencing libraries were measured and checked for fragment size and concentration. Libraries were considered appropriate if fragments were sized between 350 and 500 bp (resulting from the previous cDNA fragment having adapters added). The samples were sequenced with Illumina HiSeq 2000 equipment and analyzed using CLC Genomics Workbench software. Sequence reads were checked for quality and alignment to known transcripts. At least 16000 transcripts were identified in all groups. Quantitative and qualitative information of the transcripts are still being processed. Based on the methodology described in which a very small number of embryos is necessary for quantifying thousands of transcripts, we conclude that RNA-Seq can be an important tool for studies in several areas of assisted reproduction such as embryo development, health, and genetic markers among others.

**Acknowledgments:** FAPESP and Gertec Embriões.



A259 Supporting Biotechnologies

## Ultrasound features of the corpus luteum on the day of transfer and pregnancy diagnosis of bovine recipients: preliminary results

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**Keywords:** cattle, colour Doppler, FTET.

The goal was to evaluate ultrasound (US) characteristics – B and color Doppler mode (CD) - of the CL of embryo recipients on day 7 of the estrous cycle, relate the results with pregnancy diagnosis at day 35 (PD35) and compare the size of CL observed in B mode with the subjective dimensions of the CL by rectal palpation. Sixty-one crossbreed recipients (*Bos taurus* x *Bos indicus*) received frozen embryos (Ethylene glycol 1.5 mol) – produced in South Africa (Embryo Plus, Brits) - after hormone protocol for timed embryo transfer (TET) – estrous synchronization (D0) and embryo transfer (D7). On D7, the CL was classified according to its size and blood flow: score 1 (Large; High blood flow in most of the gland), 2 (Medium; Regular or Concentrated blood flow in certain areas of the gland) and 3 (Small; Poor or low blood flow), considering the size difference between the ovaries. The CL was also evaluated by rectal palpation (PR) in large, medium and small. All procedures were performed by the same US operator. Approximately 400 frames in B mode and 100 in color-flow Doppler, containing entire cross-sections of the CL, were stored in avi format. The area of the CL at largest diameter and averaged area of the CL with colored pixels - indicative of blood flow – from three regions (central and opposite sides of the CL) were measured with ImageJ software (Image Processing and Analysis in Java). The CL and blood flow areas were compared to scores for size (PR and B mode) and blood flow (CD). The PROC GLM procedure of SAS statistical software (version 9.0, SAS Institute Inc., Cary, NC, USA.) and the Duncan test were used to access the differences among means. Pregnancy diagnosis was performed on D35 and PROC FREQ was used to separate pregnant (G) of non-pregnant (NG) animals within the CL scores of D7. The pregnancy rate at 35 days was 50.8% (31/61), and it was similar to previous described for frozen embryos from *in vivo* production (Fernandes, 1999, ABMVZ, 51, 263-66). The subjective scores for size (PR and B mode) and blood flow of the CL, given on D7, were not efficient ( $P < 0.5$ ,  $< 1.0$  and  $< 0.4$ , respectively) to predict G and NG animals. Luteal and blood flow areas on D7 did not differ ( $P < 1.0$ ,  $< 0.6$ , respectively) between animals later (DG35) diagnosed as G ( $320.4 \pm 64.6$  and  $55.9 \pm 19.0$ , respectively) and NG ( $321.3 \pm 71.2$  mm<sup>2</sup> and  $53.9 \pm 21.5$  mm<sup>2</sup>, respectively). The measured CL area was similar ( $P > 0.05$ ) among RP scores for CL size. However, the B mode scores were more efficient ( $P < 0.0001$ ) to separate CLs of different sizes ( $356.4 \pm 68.2a$ ,  $316.3 \pm 50.7a$  e  $235.6 \pm 33.5b$  mm<sup>2</sup>, respectively for scores 1, 2 and 3). The real time observation CL in CD mode was sensitive ( $P < 0.0001$ ) to detect blood flow changes of the CL tissue ( $66.8 \pm 18.4a$ ,  $45.2 \pm 15.7b$  e  $38.4 \pm 16.1b$  mm<sup>2</sup>, respectively for CD scores 1, 2 and 3). The interpretation is that the B mode was more efficient than the PR to differentiate corpus luteum of different sizes. The combined results of B and CD modes of the ultrasound give valuable information about size and blood flow of the CL, but were not efficient to predict the chances of the D7 recipient to become pregnant.

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A260 Supporting Biotechnologies

### **Cryopreservation of somatic cells of *Alouatta fusca*, *Cervus elaphus* and *Leopardus pardalis* using DMSO and PG as cryoprotectants**

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**Keywords:** cell culture, cryopreservation, wild animals.

The cell cryopreservation is an important tool for genetic material preservation from endangered animals, for their long-term use. The formation of genetic resources includes gametes, embryos and cells (Holt and Pickard 1999 Reviews of Reproduction 4, 143-50) facilitating the creation of a genetic pool of endangered species. The aim of this study was compare survival, viability and injury in DNA, in wild animals cells cryopreserved with DMSO or propylene glycol (PG). Muscle tissue samples of *Alouatta fusca* (baboon), *Cervus elaphus* (deer) and *Leopardus pardalis* (ocelots) received in Veterinary Hospital - CAV/UDESC, obtained post mortem, were collected aseptically and transported to the laboratory. Cells were obtained by explant technique (Freshney 2005, Culture of Cells 175-197) and maintained in DMEM + 10% FCS at 37 °C with 5% CO<sub>2</sub>. The cells were cryopreserved in cryovials at a concentration of 1x10<sup>6</sup> cells/mL using a solution with 10% PG or DMSO in culture medium. After wadding, the cryovials were placed in Mr. Frosty® apparatus (Nalgene Nunc Cooler, USA) which was kept at 4 °C for 15 min and for 12 h at -80 °C. Then the cryovials were transferred to N<sub>2</sub>L. For thawing, cryovials were kept in the water bath at 37 °C and the contents transferred to a tube containing 2 mL of culture medium, centrifuged and resuspended in 500 µL of medium. One sample was used for determination of survival by trypan blue staining. In the remainder, 10.000 cells were cultured in well with 1.9 cm<sup>2</sup> in triplicate and them viability was assessed by counting the number of living cells after 24 h of cultivation. Another part of the samples were used for the comet assay (duplicate) (Collins 2004, Molecular Biotechnology 26, 249-261). In all tests cells not cryopreserved were used as control. Statistical analyzes were performed using SAS (SAS Institute, 2000), PROC GLM and subsequent comparison of means by Tukey test (5%). Rates of cell survival for DMSO and PG, immediately after thawing, were respectively, 81.2% and 71.8% (baboon), 68.8% and 68.8% (deer), 68.7% and 69.1% (ocelot) no significant difference in the same animal. The viability after 24 h of culture cells from the same animal, frozen with different cryoprotectants showed no significant difference (P<0.05), and during this time, the increase of the cell number for DMSO and PG were respectively, 43.3% and 46.7% (baboon), 50.0% and 53.3% (deer), 22.5% and 22.5% (ocelot). The DNA integrity assessed by comet assay showed no difference between the categories of damage when compared to cells frozen with DMSO or PG, with respectively 74.5% and 77.5% (baboon), 68.5% and 75.5% (deer), 64.5% and 67.5% (ocelot) showed no injury, as well as controls baboon (85%), deer (82%) and ocelot (80%). Therefore, the use of DMSO or PG in cryopreservation of cells these animals did not affect the cell viability by parameters analyzed.



A261 Supporting Biotechnologies

### Identification of CAE virus by qPCR in embryos recovered from seropositive dairy goats

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**Keywords:** caprine, CEA, real time PCR.

The aim of the present study was to identify by qPCR the presence of the CAEV (caprine arthritis-encephalitis virus) in goat embryos produced in vivo following embryo washes recommended by the IETS, 1998 (Stringfellow, DA, Seidel, D.A., Manual International Embryo Transfer Society). Embryos were flushed according to Fonseca, J.F. et al., 2012 (In press) from ten (n=10) dairy goats aged 4 and 5 years naturally infected and positive for CAE virus by AGID test. Subsequently embryos with intact ZP (morule and initial blastocysts) were divided into two groups; the washed embryos (n = 44) and unwashed embryos (n = 44), totaling 44 pools. The RNA of two embryos pooled from the same flushing was extracted using the MinElute Virus Spin Kit® (Qiagen, Düsseldorf, USA). After that the material was subjected to Real Time PCR, the positive control was goat synovial membrane. All samples were negative confirming the results reported by Ahmad et al., 2008 (Theriogenology, 69, 408-415) where the presence of CAE virus in goat embryos produced in vivo was not detected by conventional PCR. The present study shows that embryos derived from CAE-positive animals are free of the pathogen independently of washing. However, considering embryo commercialization rules the recommended washes must be maintained. Further studies are being carried out to determine the risk of transmission of pathogens in naturally infected animals, in order to achieve the international trade of embryos from CAE-positive goats.



A262 Supporting Biotechnologies

### Adaptation and evaluation of negative pressure effects on pre-freezing of ram semen

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**Keywords:** Controlled stress, cryopreservation, heterologous IVF.

The induction of controlled stress by positive pressure increases the cryotolerance of mammal gametes and embryos. However, the device to apply positive pressure is difficult to obtain and to transport. Our group showed that the negative pressure obtained in a low cost and easy to handle equipment (Nitrocooler) has similar effect to positive pressure. The aim of this study was to evaluate the effect of 5 min negative pressure treatment of ram semen, before freezing. Semen was pooled from ejaculates of 2 rams collected with the aid of an artificial vagina, and diluted 1 + 1.5 in Tris-yolk medium, fractionated into 4 aliquots and submitted to: control; pressure of 200 mBar (P200); pressure 500 mBar (P500) and pressure of 800 mBar (P800). Just after, an identical fraction containing 10% glycerol was added to the each aliquot, and loaded in 0.25 mL straws, cooled and frozen in a TK3000 Compact machine (5 replications). Post-thaw evaluations performed were: progressive motility (PM) just after thaw and during the Thermo-resistance-test - TRT (1, 2, and 3 h) evaluated by optical microscopy; acrosomal integrity (AI) by FITC staining; membrane integrity (MI) through the hiposmotic test, PM after selection by percoll (PMPP), acrosome integrity post percoll (AIPP), membrane integrity after percoll (MIPP) and cleavage rate after heterologous IVF with bovine oocytes. Data were analyzed as a randomized block design, with no effect of repetition, by Fischer LSD tests with 5% significance. Just after thaw the PM of Control group (49±7.4) was higher than P200 (40.9±9.7), P500 (38.9±7.4) and P800 (38.9±7.4) that did not differ among them. There were no differences in PM between Control, and P200, P500 and P800 groups during the TTR 1 h (43.4±9.7; 37.9±7.5; 36.9±7, and 37.9±7, respectively), 2 h (40.8±8.6; 31.8±6; 34.8±7.7 and 36.8±8.7, respectively) and 3 hours (34.8±10; 28.7±8.2; 33.8±9.7 and 29.8±16 respectively), and yet in AI (55.4±13.2; 52.4±5.8; 49.5±7 and 49.8±11.4 respectively), PMPP (66±4.1; 59.2±10.2; 51.7±19.2 and 63.2±9.1, respectively), AIPP (46, 4±4.6; 45.7±4.1; 46.2±5.7; 49.3±8.9, respectively) and MIPP (45.4±10.2; 39.9±4.9; 47.3±9.4 and 41.1±8.9, respectively). For MI evaluation the Control group (36.6±7.7) was higher than P200 (30.2±5.8) and P800 (30.4±6.6) but did not differ from P500 group (34.3±7). Regarding cleavage, the P800 group (34.5) was lower than all other groups (control 44.3, P200 51.2 and P500 50.9) that did not differ among them. Data show that even with an initial reduction in PM, as the time passed the negative effects of P200 and P500 pressures disappeared. However, it was still observed a negative effect of P800 pressure. We concluded that the P200 and P500 applied to the ram semen do not reduce its viability after freezing, being necessary to evaluate a possible effect in pregnancy rates after the use of negative pressure treated semen.



A263 Supporting Biotechnologies

### **Uterine blood flow in bitches with cystic endometrial hyperplasia-pyometra**

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**Keywords:** bitch, diagnosis, Doppler.

The Cystic Endometrial Hyperplasia (CEH)-Pyometra Complex comprises a group of uterine diseases named CEH-mucometra, endometritis and pyometra. The differential diagnosis of these diseases is accomplished through the combined results of two-dimensional ultrasound, clinical signs and uterine macroscopic and histological evaluation. The aim of this study was to characterize the uterine blood flow in bitches with distinct uterine pathological conditions in order to develop a noninvasive and early method of diagnosis. We allocated 28 bitches into 3 groups, according to clinical signs, ultrasound analysis and uterine histological examination (H/E stain): Control-Diestrus Group (n=6), CEH-Mucometra Group (n=10) and Endometritis-Pyometra Group (n=12). With the use of colour Doppler, the uterine vessels were identified for quantitative assessment of the overall uterine vascularization and the pulsed-wave Doppler was employed to classify the flow velocity waveforms. Parameters of blood flow velocity (PS, ED and TAMAX) and hemodynamic parameters (RI, PI and S/D) were calculated by the ultrasound software. A total of nine stable uterine artery waves was obtained to calculate the average for each variable. The degree of uterine vasculature was subjectively scored as 1 to 4, being 1 the minimum and 4, the maximum degree. Data were compared by ANOVA and LSD ( $p \leq 0.05$ ). Control (PS:  $47.3 \pm 2.6$ ; ED:  $1.3 \pm 0.5$ ; TAMAX:  $8.5 \pm 1.1$ ) and CEH-Mucometra (PS:  $60.8 \pm 4.5$ ; ED:  $9.8 \pm 0.8$ ; TAMAX:  $20.5 \pm 0.1$ ) groups showed lower blood flow velocities when compared to Endometritis-Pyometra Group (PS:  $86.7 \pm 6.3$ ; ED:  $33.3 \pm 3$ ; TAMAX:  $48.3 \pm 4.1$ ). Conversely, Control (RI:  $0.97 \pm 0.01$ ; PI:  $6.4 \pm 0.6$ ; S/D:  $78.8 \pm 9.8$ ) and CEH-Mucometra (RI:  $0.8 \pm 0$ ; PI:  $2.6 \pm 0.1$ ; S/D:  $6.7 \pm 0.4$ ) groups presented higher hemodynamic indices in comparison to Endometritis-Pyometra Group (RI:  $0.6 \pm 0.02$ ; PI:  $1.2 \pm 0.1$ ; S/D:  $2.9 \pm 0.2$ ). In the latest Group, uterine perfusion was characterized as continuous blood flow. The quantitative uterine vascularization was: Control Group - minimum and maximum of score 1, CEH-Mucometra Group - minimum of score 1 and maximum of score 2; Endometritis-Pyometra Group - minimum of score 2 and maximum of score 4. Our study demonstrated that the severity of the uterine disease is associated with higher blood flow, fundamental in the compensatory mechanisms against the infectious agent. Doppler velocimetric evaluation may allow for the differential diagnosis between CEH-Mucometra and Endometritis-Pyometra, whenever clinical symptoms and ultrasonographic findings are inconclusive. Thus, when the two-dimensional ultrasound evaluation does not allow differential diagnosis, analysis of hemodynamic indices (RI, PI and S/D) of the uterine artery can be complementary. We conclude that Doppler ultrasound is a noninvasive diagnostic method, with high sensitivity and specificity for the differential diagnosis of HEC-mucometra-Endometritis and Pyometra in bitches.



A264 Supporting Biotechnologies

### **Effect of the addition of pentoxifylline to the semen extender on seminal characteristics of stallion thawed semen**

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**Keywords:** CASA, membrane, spermatozoa.

Pentoxifylline, a methylxanthine derivate, is an inhibitor of the enzyme phosphodiesterase, that increase the intracellular cAMP concentration. The pentoxifylline stimulates flagellar motility therefore increasing sperm motility (STANIC et al., 2002, Intern J Androl, 25, 186-90). The purpose of this study was to examine the effects by adding pentoxifylline to seminal extender on cryopreserved equine semen after thawing. Eight batches from a unique stallion was cryopreserved ( $100 \times 10^6$  spermatozoa/0.5 mL straws). Two semen straws were thawed and divided in two aliquots. One of the aliquots was diluted in skim milk extender, the other aliquot was diluted in the same skim milk extender plus pentoxifylline (7.18 mM). Aliquots of each treatment were warmed in a water bath at 37°C and analyzed at different time after dilution: T0 (5 min), T30 (30min), T60 (60 min) e T120 (120 min). The sperm movement was evaluated using the Computer-Assisted Semen Analysis (CASA), integrity of plasma and acrosomal membranes and mitochondrial membrane potential, using the fluorescent probes association (PI, H342, FITC-PSA and JC-1) and chromatin denaturation accessed by Toluidine blue. To all variables was utilized the analysis of variance (ANOVA), and the range was compared by *LSD* test, SAS 9.3 version (2010). For statistical analyses, it was considered effect of treatment, time and treatment x time interaction. No improvement was found on total motility, progressive motility, beat cross frequency (BCF), and percentage of rapid cells in none of the analyzed periods. However, pentoxifylline increased ( $P < 0.05$ ) VAP ( $71.68 \pm 1.52 \times 65.71 \pm 0.89 \mu\text{m/s}$ ), progressive velocity (VSL,  $63.63 \pm 1.29 \times 59.20 \pm 0.80$ ), curvilinear velocity (VCL,  $131.24 \pm 2.51 \times 118.50 \pm 1.57 \mu\text{m/s}$ ) and displacement lateral of head (ALH;  $5.17 \pm 0.09 \times 4.69 \pm 0.07 \mu\text{m}$ ) when compared to controls. Pentoxifylline decreased ( $P < 0.05$ ) straightness (STR;  $88.61 \pm 0.40 \times 89.67 \pm 0.27\%$ ) and linearity (LIN;  $51.45 \pm 0.49 \times 53.45 \pm 0.45\%$ ). Membrane integrity wasn't affected by pentoxifylline addition and it did not provide any protective behavior in the length of incubation. Chromatin integrity also was not affected by extender, neither the time of incubation. In conclusion, pentoxifylline increase some motility parameters but it does not affect cellular membranes or chromatin membrane.

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A265 Supporting Biotechnologies

### Bovine embryo genotyping on high-throughput SNP platform

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**Keywords:** biopsy, marker-assisted selection, WGA.

Despite the great development of animal reproduction biotechnology, such as embryo *in vitro* production (IVP), preimplantation genetic diagnosis (PGD) is still applied with restraint in animals embryo transfer. Recent advances in genomics have associated phenotypic characteristics with molecular information, allowing the development of marker-assisted selection. The aim of this study was to perform PGD in bovine embryos using high-throughput SNP platform (BeadChip/6,909 SNP). The small amount of genomic DNA (gDNA) obtained from embryo biopsy is the main limitation for the high-density SNP analysis. Thus, the Whole Genome Amplification (WGA) (Repli-g Mini Kit, Qiagen, Hilden, Germany) was used to increase the amount of gDNA from embryo biopsy and allow the analysis of thousands SNP simultaneously. Eighty-eight IVP bovine embryos were subjected to micromanipulation by microaspiration, allowing the formation of three groups with different numbers of biopsied cells: G1) 5-10 (n=28); G2) 10-20 (n=37); G3) > 100 - hatched blastocyst (n = 23). All samples were subjected to the same WGA protocol, and 4µL of each sample were used for genotyping on iScan/Illumina platform. The genotyping quality was assessed using the Call Rate (CR), GenCall Score (GC10), Allele Drop In (ADI) and Allele Drop Out (ADO). Kruskal-Wallis test was applied to investigate differences in the distribution of variables among groups. Spearman's rank correlation coefficient revealed a significant correlation between all variables. The results showed a positive correlation between CR and GC10 (0.99/P <0.001), while ADI and ADO rates were negatively correlated with CR and GC10 (ADI/CR: -0.87; ADI/GC10: - 0.88; ADO/CR: -0.87; ADO/GC10: -0.86), P<0.001 for all variables. Kruskal Wallis pointed to significant differences in all variables (CR, GC10, ADO and ADI) among the 3 groups of biopsies (G1, G2 and G3). The CR average was 59.26%, 78.47% and 95.97% for G1, G2 and G3, respectively. It was developed a script (mendellFix) based on the "Law of Segregation", for inference of not determined genotypes based on the parents genotypes, thus increasing the CR of group 1, 2 and 3 to 79.69%, 88.20% and 97.28%, respectively. The results of this study show that it is possible to perform the genotyping of bovine embryos in high-throughput SNP platform with samples subjected to WGA/MDA protocol, but the number of cells obtained by embryo biopsy affects the quality of genotyping. The association of biotechnologies described in this work allows the application of marker-assisted selection in bovine embryo, contributing to further accelerate the process of animal breeding.



A266 Supporting Biotechnologies

### Active immunization of cows in the pre-partum period and transfer of passive immunity to newborn calves for bovine viral diarrhea virus and bovine herpesvirus

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**Keywords:** BoHV-1, BVDV, vaccine.

The aim of this research was to evaluate the humoral immune response of the commercial vaccine Cattle Master Gold FP5 + L5 (Zoetis<sup>®</sup>, Madison, USA) for BVDV and BoHV-1 in the pre-partum of Holstein cows, as well as the transfer of specific antibodies (AB) from mothers to their calves for passive immunity. Cows screened (n=11) were negative in the serum neutralization test (SN) for BVDV and BoHV. Of these, 6 were immunized by subcutaneous injection of 5 mL of vaccine, which contains 5960 strain of BVDV-I, 53637 of BVDV-II inactivated and RLB103 of BoHV-1 attenuated at 42 and 21 days (d) of the expected delivery date. Control unvaccinated cows were maintained (n=4). Calves were fed with six liters of colostrum from their mothers in the first 12 hours of life. Blood samples were collected from mothers in three moments: M0 - before the 1st dose, M1 - before the 2nd dose, M2 - 21 d after the 2nd dose. The samples of calves were obtained before (T0) and after feeding of colostrum at 48 hours of life (T1). The measurement of neutralizing ABs in mothers and calves by SN for BVDV and BoHV followed similar protocols (OIE 2010, Terrestrial Manual, 1-17). The frequency of seropositivity for BVDV-1 and BoHV in the group of immunized mothers was 33.33% (2/6) and 100% (6/6), respectively. The mothers of the non-vaccinated group remained seronegative. All calves in this study showed no evidence of antibodies to BVDV and BoHV-1 at T0, however, it was possible to detect seroconversion of newborns from seropositive mothers after active immunization for BVDV and BoHV-1. Geometric mean titers (GMT) obtained in positive mothers in SN was zero in M0, 16 in M1 and 64 in M2 to BVDV, and M0=0, M1=13.45 and M2=71.52 for BoHV-1. The GMTs of calves in T1 were 32 to 77.71 for BVDV and BoHV-1. Thus, maternal vaccination during the pre-partum period induced the arising of neutralizing ABs in mothers, especially to BoHV. Moreover, the transfer of maternal ABs to newborns was detected after colostrum feeding. It is worth noting that only inactivated BVDV vaccines can be commercialized in Brazil, however, these have lower biological immunogenicity when compared to live vaccines. Previous study (Silva, 2006, Federal University of Santa Maria, 35-55) evaluated six Brazilian commercial vaccines for BVDV and only 1/6 was able to induce neutralizing antibodies in vaccinated cattle. Thus, despite the lower response to BVDV compared with BoHV in this study, the seroconversion rate obtained was better than those obtained in the aforementioned study. In addition, the immune response of the mothers and calves should not be evaluated only for the production of neutralizing ABs, since the immunogen must also confer cellular immunity. Thus, studies are being conducted to evaluate the lymphocyte subpopulations, as well as its activation, and this information is essential to complement the data obtained and preliminarily presented.



A267 Supporting Biotechnologies

### **Pregnancy monitoring of vitrified *in vitro* produced embryos in buffaloes**

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**Keywords:** birth, buffaloes, vitrification.

Vitrification has proven more appropriate technique of cryopreservation of mammalian *in vitro* embryos. However, even with several studies being conducted in the species, there are few trials evaluating pregnancy and calving rates after embryo transfer (ET) (Presicce, 2007, *Reprod Domest Anim*, 42, 24–32). For this reason, the aims of the present study were to monitor pregnancy after transfer of vitrified embryos and to collect data of calves and recipients after delivery. Expanded blastocysts grade I, produced *in vitro* (IVP) from ovun pick up (OPU) buffaloes donors were vitrified by the cryotop method (De Rosa et al., 2007, *Ital J Anim Sci*, 6, 747-50) on days 5, 6 and 7 of culture. At eight sessions, 70 vitrified embryos were transferred in the period of 01/02/11 to 02/04/12 to recipients previously synchronized at day 6 after ovulation, utilizing embryo transfer fixed time protocol (Baruselli et al., *Rev Bras de Reprod Anim*, 31, 285-92). All embryos were warmed in the lab (De Rosa et al., 2007, *Ital J Anim Sci*, 6, 747-50) and loaded into straws for ET and there weren't temporary culture in CO<sub>2</sub> incubators or selected. On day 30 after ET, positive pregnancy was diagnosed in 26 animals (37.1%). A total of 9 fetal mortalities (34.6%) were verified from 30 days until calving, in which 4 occurred from 30 to 60 days after ET (15.4%), and 5 occurred from 60 days until calving (19.2%). In this last period, 5 pregnancy losses were due to abortion. From these 17 pregnancies, a total of 10 female calves (58.8%) and 7 male calves (41.2%) were born. Pregnancies from female and male calves had a mean length of 313.2 and 316.4 days, respectively (range 302 to 332 days, and 311 to 323 days, respectively). Weight at calving was a mean of 33,6 and 34 kg for female and male calves, respectively (range 30 to 38 kg, and 32 to 35 kg, respectively). All calving occurred without intervention and dystocia was observed in only one case. No large offspring syndrome, hydramnios, hydroallantois, or umbilical cord anomalies were observed in calves. Delivery was normal and without induction in all recipients, and any puerperal infections, or retained placenta occurred. Suckling assistance was necessary in only one newborn. Results are unprecedented in science and opens possibilities for the commercial use of the techniques of OPU, IVP and ET of vitrified embryos in buffaloes.

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Dias BL	<u>616</u>	Fernandez-Curi E	<u>562</u>	Garcia JM	<u>493, 521, 531</u>
Dias CC	<u>206</u>	Ferraz ALJ	<u>568</u>	Garcia RF	<u>453</u>
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Dias LMK	<u>466</u>	Ferreira AMR	<u>615</u>	Garcia-Ispierto I	<u>252</u>
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Diaz DJ	<u>510</u>	Ferreira AR	<u>396, 613</u>	Gasparrini B	<u>635</u>
Diniz JPC	<u>629</u>	Ferreira CER	<u>461</u>	Gasperin BG	<u>461</u>
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Diógenes MN	<u>372, 396, 525, 535</u>	Ferreira MEO	<u>619, 620, 627</u>	Gastal GDA	<u>461</u>
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Dubeibe DF	<u>406</u>	Folchini NP	<u>499</u>	Giotto AB	<u>498, 505</u>
Duchi R	<u>334, 365</u>	Fonseca JF	<u>420, 533, 629</u>	Giroto RW	<u>432, 476, 483, 398, 410,</u>
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Duran MS	<u>437</u>	Fontes PK	<u>407, 424, 462</u>	Glanzner WG	<u>383, 416, 554, 577, 585, 587</u>
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<b>F</b>				Gomes MGT	<u>519</u>
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Guido SI	<u>450</u>	Laus AC	<u>569</u>	Lunardon NT	<u>422</u>
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Guimarães ALS	<u>495, 535, 613</u>	Lazzarotto CR	<u>419</u>	<b>M</b>	
Guimarães CF	<u>594</u>	Leal CLV	<u>409, 418, 517, 575, 583, 595, 625</u>	Macari RC	<u>443, 479</u>
Guimarães CRB	<u>215, 619, 620, 627</u>	Leão BCS	<u>589, 590, 617</u>	Macedo GG	<u>401, 411, 431, 440, 444, 458, 470</u>
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Nogueira FF	<u>441</u>	Ortega MS	<u>556</u>	Pinto VSC	<u>634</u>
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Oliveira ACS	<u>460</u>	Parra NC	<u>518</u>	Prata AB	<u>199, 435, 480, 492</u>
Oliveira AP	<u>629</u>	Paschoal DM	<u>160, 368, 508, 542</u>	Price C	<u>400, 423</u>
Oliveira BM	<u>557, 632</u>	Paula FBE	<u>515, 518</u>	Puelker RZ	<u>576</u>
Oliveira CA	<u>466</u>	Paula NG	<u>551</u>	Pugliesi G	<u>470, 481, 558, 567, 574, 588, 599</u>
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Recalde HC	<u>632</u>	Rodríguez-Alvarez L	<u>358</u> , <u>361</u>	Santos ECS	<u>528</u> , <u>564</u>
Regazzi FM	<u>387</u> , <u>570</u> , <u>572</u> , <u>631</u>	Rodríguez-Alvarez LI	<u>552</u>	Santos ES	<u>539</u>
Reis WSM	<u>619</u> , <u>620</u> , <u>627</u>	Rodríguez-Martínez H	<u>148</u> , <u>268</u>	Santos FA	<u>483</u>
Remião MH	<u>582</u>	Rojas D	<u>358</u>	Santos FAP	<u>389</u>
Rengifo-Garrido O	<u>562</u>	Romero JJ	<u>311</u>	Santos FF	<u>466</u>
Renno FP	<u>444</u>	Rondina D	<u>419</u>	Santos Filho AS	<u>450</u>
Resende AO	<u>454</u>	Rosa CO	<u>404</u> , <u>417</u>	Santos FL	<u>571</u>
Resende HL	<u>412</u>	Rosa FQ	<u>505</u>	Santos FW	<u>499</u>
Resende MV	<u>394</u>	Rosa PRA	<u>554</u> , <u>577</u> , <u>585</u> , <u>587</u> , <u>592</u>	Santos GB	<u>559</u>
Rettore JVP	<u>610</u>	Ross P	<u>626</u>	Santos GMG	<u>180</u> , <u>404</u> , <u>411</u> , <u>417</u> , <u>451</u> , <u>452</u> , <u>512</u> , <u>534</u>
Rezende AA	<u>469</u>	Rossi DM	<u>511</u>	Santos JEP	<u>465</u>
Rezende VG	<u>516</u>	Rossi GF	<u>521</u> , <u>531</u>	Santos JHP	<u>483</u>
Ribeiro DBV	<u>392</u> , <u>439</u> , <u>532</u>	Rossi JR	<u>453</u> , <u>503</u> , <u>547</u> , <u>550</u> , <u>620</u> , <u>627</u>	Santos JM	<u>369</u> , <u>370</u>
Ribeiro Filho AL	<u>378</u> , <u>392</u> , <u>395</u> , <u>436</u> , <u>439</u> , <u>472</u> , <u>482</u> , <u>532</u>	Rossi TC	<u>438</u> , <u>459</u> , <u>514</u> , <u>516</u> , <u>547</u> , <u>550</u>	Santos JMS	<u>421</u>
Ribeiro JA	<u>537</u> , <u>548</u>	Rossini JB	<u>416</u>	Santos JT	<u>404</u> , <u>554</u> , <u>577</u> , <u>585</u> , <u>587</u> , <u>592</u>
Ribeiro Junior M	<u>477</u>	Rutigliano H	<u>354</u>	Santos LP	<u>421</u>
Ribela MTCP	<u>593</u>	<b>S</b>		Santos MM	<u>395</u> , <u>436</u> , <u>482</u> , <u>532</u>
Rigo AG	<u>496</u>	Sá AKCP	<u>509</u> , <u>630</u>	Santos RM	<u>549</u>
Rigo ML	<u>383</u> , <u>554</u>	Sá AL	<u>527</u> , <u>536</u>	Santos SDSD	<u>527</u> , <u>536</u>
Risolía PHB	<u>426</u> , <u>600</u>	Sá Filho MF	<u>349</u> , <u>411</u> , <u>415</u> , <u>470</u> , <u>477</u> , <u>478</u> , <u>481</u> , <u>496</u> , <u>506</u> , <u>561</u>	Saraiva NZ	<u>531</u>
Rissi VB	<u>582</u> , <u>585</u> , <u>587</u>	Sá Filho OG	<u>206</u>	Saravia F	<u>358</u>
Rizzo H	<u>469</u>	Sala RV	<u>401</u> , <u>411</u> , <u>444</u> , <u>446</u> , <u>474</u> , <u>478</u>	Sartor DJ	<u>423</u>
Rocha AA	<u>510</u>	Salamone D	<u>581</u> , <u>586</u> , <u>609</u> , <u>621</u> , <u>349</u> , <u>385</u> , <u>432</u> , <u>446</u> , <u>458</u> , <u>466</u> , <u>476</u> , <u>479</u> , <u>483</u>	Sartori R	<u>199</u> , <u>381</u> , <u>389</u> , <u>396</u> , <u>414</u> , <u>435</u> , <u>456</u> , <u>459</u> , <u>464</u> , <u>480</u> , <u>492</u> , <u>530</u> , <u>576</u> , <u>613</u>
Rocha AM	<u>382</u>	Sales JNS	<u>458</u> , <u>466</u> , <u>476</u> , <u>479</u> , <u>483</u>	Sarzedas AC	<u>420</u>
Rocha CLR	<u>591</u>	Saliba WP	<u>635</u>	Sato EM	<u>527</u>
Rocha JC	<u>623</u>	Sampaio RV	<u>603</u> , <u>607</u>	Satrapa R	<u>471</u> , <u>485</u> , <u>523</u> , <u>541</u>
Rocha Junior CV	<u>560</u>	Sampaio WV	<u>406</u>	Satrapa RA	<u>407</u> , <u>424</u> , <u>462</u>
Rocha-Frigoni NAS	<u>589</u> , <u>590</u> , <u>617</u>	Sanches ACS	<u>398</u> , <u>410</u> , <u>537</u>	Saura LV	<u>408</u> , <u>565</u>
Rodakiewicz SM	<u>601</u> , <u>628</u>	Sanches BV	<u>496</u> , <u>534</u>	Schiavon RS	<u>564</u>
Rodello L	<u>381</u> , <u>389</u>	Sanches CP	<u>442</u> , <u>473</u> , <u>488</u> , <u>491</u> , <u>530</u>	Schwarz KL	<u>409</u> , <u>418</u> , <u>517</u> , <u>575</u> , <u>583</u> , <u>595</u> , <u>625</u>
Rodrigues ADP	<u>206</u> , <u>429</u> , <u>447</u> , <u>457</u> , <u>467</u> , <u>473</u> , <u>486</u> , <u>491</u>	Sánchez E	<u>596</u>	Schwengber EB	<u>499</u>
Rodrigues ALR	<u>420</u> , <u>501</u>			Scolari SC	<u>567</u> , <u>574</u> , <u>599</u>
				Seber MF	<u>619</u> , <u>620</u> , <u>627</u>
				Seddon AS	<u>530</u>



Seneda MM	<u>180, 386, 399, 403, 404, 417, 422, 448, 451, 452, 500, 512, 534, 582</u>	Silveira EL	<u>396</u>	Strefezzi RF	<u>611</u>
Serapião RV	<u>501, 522</u>	Silveira JC	<u>416</u>	Sturmey RG	<u>258</u>
Serrano-Pérez B	<u>252</u>	Sinedino LD	<u>465, 501</u>	Sudano MJ	<u>160, 368, 508, 542, 576</u>
Shimoda E	<u>522</u>	Siqueira AF	<u>393, 415, 600</u>	Sudré AP	<u>425</u>
Siloto LS	<u>404, 417</u>	Siqueira LGB	<u>215, 359, 425, 515, 546</u>	Surjus RS	<u>199, 414, 435, 492, 576</u>
Silva ACS	<u>380, 563, 580</u>	Smirnova NP	<u>311</u>	<b>T</b>	
Silva AR	<u>500, 537, 548</u>	Soares CA	<u>566, 579, 597, 618</u>	Tabosa RF	<u>468</u>
Silva BB	<u>527</u>	Soares JG	<u>349, 411, 431, 440, 443, 458, 470, 474, 477, 479</u>	Tanuri A	<u>633</u>
Silva BDM	<u>372, 396, 405, 613</u>	Solto LA	<u>489</u>	Tarrago OFB	<u>385, 390, 419</u>
Silva BT	<u>634</u>	Sonoda MT	<u>618</u>	Tavares KCS	<u>419</u>
Silva CA	<u>386</u>	Sousa FC	<u>510</u>	Teixeira AA	<u>432, 446</u>
Silva CB	<u>403, 451</u>	Sousa IOT	<u>397, 475</u>	Teixeira DÍA	<u>510, 621</u>
Silva CG	<u>520, 602</u>	Sousa JF	<u>519</u>	Teixeira LB	<u>537</u>
Silva CMG	<u>419</u>	Sousa Junior A	<u>397, 475</u>	Teixeira WT	<u>448</u>
Silva CPC	<u>634</u>	Sousa RV	<u>613</u>	Thatcher WW	<u>465</u>
Silva DC	<u>441</u>	Sousa SRS	<u>397, 475</u>	Thomé HE	<u>557</u>
Silva FLM	<u>389, 480</u>	Souto PP	<u>450</u>	Ticianelli JS	<u>576</u>
Silva GN	<u>369, 370</u>	Souza AH	<u>349</u>	Tirapelli ACN	<u>494</u>
Silva JCB	<u>489</u>	Souza AK	<u>399, 403</u>	Toma CDM	<u>389</u>
Silva JF	<u>528</u>	Souza AP	<u>507</u>	Torreão JNC	<u>571</u>
Silva JRV	<u>605</u>	Souza DC	<u>440, 443, 458, 474</u>	Torres A	<u>358</u>
Silva Junior RA	<u>373</u>	Souza ED	<u>359, 515, 518</u>	Torres Filho RA	<u>615</u>
Silva LA	<u>412, 481, 557, 599, 606, 615</u>	Souza EDF	<u>446</u>	Torres FP	<u>484</u>
Silva LCG	<u>387, 570, 572, 631</u>	Souza FB	<u>601</u>	Torres MA	<u>371, 375, 379, 384, 388</u>
Silva LFMC	<u>394</u>	Souza FC	<u>386, 448</u>	Torres NF	<u>406, 616</u>
Silva LG	<u>509, 630</u>	Souza FS	<u>464</u>	Trindade AB	<u>493</u>
Silva LM	<u>419</u>	Souza HM	<u>377</u>	Tsunoda RH	<u>632</u>
Silva MR	<u>456, 459</u>	Souza JAT	<u>397, 475</u>	<b>U</b>	
Silva PRL	<u>463, 497, 504</u>	Souza JM	<u>561</u>	Urio M	<u>601, 608</u>
Silva RB	<u>391</u>	Souza JS	<u>573</u>	Utsunomiya YT	<u>297, 633</u>
Silva RCP	<u>477</u>	Souza LC	<u>380, 563, 580</u>	<b>V</b>	
Silva RF	<u>536</u>	Souza MB	<u>413</u>	Vale WG	<u>433, 468, 578</u>
Silva RT	<u>380, 563, 580</u>	Souza RT	<u>414</u>	Valentim R	<u>466</u>
Silva SV	<u>377</u>	Souza SMP	<u>614</u>	Valle RV	<u>414</u>
Silva T	<u>566, 579, 597</u>	Souza-Fabjan JMG	<u>524</u>	Van Hoeck V	<u>258</u>
Silva TH	<u>549</u>	Sovernigo TC	<u>511, 544, 551</u>	Van Tilburg MF	<u>622</u>
Silva TVG	<u>527, 536</u>	Spencer TE	<u>239</u>	Vannucchi CI	<u>373, 374, 387, 570, 572, 631</u>
Silva WAL	<u>413</u>	Spilimbergo E	<u>621</u>	Vantini R	<u>521, 531</u>
Silva-Santos KC	<u>180, 404, 417, 422, 451, 452, 512, 534</u>	Spilmann I	<u>628</u>	Vanzin JÁ	<u>468</u>
Silveira CRA	<u>437, 463, 497, 502, 504</u>	Sponchiado M	<u>567</u>	Varago FC	<u>438, 494, 514, 516, 547, 550, 559</u>
		Spricigo JFW	<u>396, 405, 525</u>		
		Staub JV	<u>464</u>		
		Stefano E	<u>513</u>		



Vargas MW	<u>559</u>	Viana JHM	<u>215, 359, 420,</u> <u>425, 438, 493,</u> <u>514, 515, 518,</u> <u>526, 546, 610,</u> <u>619, 620, 627</u>	Welchek NM	<u>433, 468</u>
Vargas TC	<u>584</u>	Viau P	<u>466</u>	Wheeler MB	<u>351, 355, 356</u>
Vasconcelos CGC	<u>467</u>	Vicente WRR	<u>533</u>	White KL	<u>354</u>
Vasconcelos FS	<u>486</u>	Viechnieski S	<u>484</u>	Wicke AA	<u>377</u>
Vasconcelos JLM	<u>206, 395,</u> <u>429, 442,</u> <u>447, 457,</u> <u>467, 473,</u> <u>486, 488, 491</u>	Viegas D	<u>528</u>	Wiltbank MC	<u>206, 414, 435, 442,</u> <u>457, 473, 480, 488</u>
Vasenlow J	<u>568</u>	Vieira AD	<u>461, 528, 564</u>	Winger QA	<u>416</u>
Vater A	<u>430, 455</u>	Vieira CA	<u>598</u>	Wohlres-Viana S	<u>425, 546</u>
Veiga GAL	<u>374, 387,</u> <u>570, 572, 631</u>	Vieira LM	<u>401, 411, 463,</u> <u>476, 483, 496,</u> <u>497, 502, 504,</u> <u>506</u>	<b>X</b>	
Velásquez A	<u>361, 552</u>	Vieira MR	<u>438</u>	-----	
Velho JC	<u>601</u>	Visintin JÁ	<u>393, 415, 543,</u> <u>600, 624, 633</u>	Xavier GS	<u>578</u>
Veraguas D	<u>361, 552</u>	<b>W</b>		<b>Y</b>	
Veras GA	<u>450</u>	-----		-----	
Veras MB	<u>442, 473,</u> <u>484, 530</u>	Wang Z	<u>354</u>	Yamakawa FHS	<u>601</u>
Vergara LE	<u>542, 508</u>	Webb BT	<u>311</u>	<b>Z</b>	
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				Zaffalon FG	<u>390, 409, 418, 517,</u> <u>575, 583, 595, 625</u>
				Zago FC	<u>555, 630</u>
				Zanin R	<u>539, 544</u>
				Zanóbia LF	<u>537, 548</u>
				Zanóbia RF	<u>548</u>
				Zuelow SJ	<u>555</u>