Recent advances in ovulation synchronization and superovulation in dairy cattle

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Introduction

The reproductive physiology of dairy cattle has been dramatically changing during the last few decades, perhaps due to increased feed intake associated with increased milk yield (1). High milk production is associated with lower circulating steroids and a decline in estrus behavior and fertility (2). In order to diminish these problems, new strategies for ovulation synchronization have been developed for both artificially inseminated and superovulated (SOV) cows. These strategies allow the use of fixed-time AI (FTAI) without the need of estrus detection. Although a significant improvement in service rate was achieved with the use of FTAI, low conception rates (CR), or low good quality embryo yield may still occur. There is substantial opportunity still available to optimize the outcomes from these protocols. This short article presents data of recent protocols that have improved CR as well as good quality embryo yield in dairy cows. Moreover, some other strategies that have been tested to improve SOV responses and embryo yield in dairy cattle are also discussed.

Recent advances in protocols for fixed-time AI in dairy cows

Previous studies showed the ideal phase to initiate the Ovsynch FTAI protocol was Day 5 to 12 of the cycle (3,4). Pre-synchronization strategies are being developed to optimize stage of the cycle at Ovsynch initiation and improve success with FTAI, particularly in anovular cows. Ovsynch produces ovulation at second GnRH treatment in both ovular and anovular cows (5); although, size of the ovulatory follicle and hormone levels are variable and not optimized. To improve pre-synchronization, a Double-Ovsynch protocol was developed, using Ovsynch for pre-synchronization followed 7 d later by a normal Ovsynch 56-FTAI protocol. In one study using 337 Holstein cows, the use of Ovsynch for pre-synchronization instead of two injections of PGF2α 14 d apart improved CR in primiparous (65 vs 45%), but not multiparous (38 vs 39%) cows (6). In another study with 514 cows, a Double-Ovsynch protocol with high progesterone (GnRH-7d-PGF2α-3d-GnRH-7d-GnRH-7d-PGF2α-56h-GnRH-16h-AI) improved CR as compared with a low progesterone protocol (GnRH-7d-PGF2α-3d-GnRH-7d-PGF2α-56h-GnRH-16h-AI), independent of parity (48 vs 33%; 7).

Recent advances in protocols for superovulation in dairy cows

Due to the delayed time for ovulation in high-producing dairy cows after luteolysis (8), studies have been performed in order to evaluate the effect of delaying the time of intravaginal progesterone implant removal, LH injection, and AI after PGF2α treatment on SOV response and embryo production in lactating Holstein cows (9). The protocol P36LH60 (progesterone implant removal at 36 h and LH injection at 60 h after PGF2α injection) increased the number of viable embryos by approximately two times as compared to conventional SOV protocols. Another recent study evaluated the effect of semen source (conventional vs pooled) and dose of FSH (200 vs 400 mg) on SOV response and embryo production in Holstein heifers that received a single AI 12 h after GnRH (10). A similar fertilization rate (83 vs 77%) and percentage of transferable embryos (52 vs 45%) recovered per flush were found for pooled high-fertility and commercial semen. The higher dose of FSH increased SOV response (16.8 vs 12.9 follicles >8 mm); however, the number (2.3 vs 3.3) and percentage (38 vs 60%) of transferable embryos did not differ between high and low FSH. In addition, lowering the dose of FSH to 200 mg decreased the number of unfertilized ova (1.6 vs 0.6).
Thus, optimizing hormonal levels during FTAI or SOV can improve success with these protocols.

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Recent advances in ovulation synchronization and superovulation in buffalo

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Introduction

Associated to the enhancement in reproductive efficiency, techniques used to achieve genetic improvement make possible to obtain herds with better productive characteristics, such as growth rate, carcass quality, milk yield, food conversion and precocity, among others. Thus, multiplication of superior animals by using reproductive biotechniques [artificial insemination (AI) and embryo transfer ET)] can provide greater economic return. Moreover, increased reproductive rates associated to genetic improvement must be the main objective of farmers to improve buffalo productivity and farms income. Therefore, the objective of this presentation is to discuss some strategies that have been tested to increase the AI and ET responses in buffalo.

Recent advances in protocols for fixed-time AI in buffalo

Artificial insemination in buffalo has limited use worldwide due the difficulties in the estrus detection and in finding an adequate moment for this procedure (Baruselli *et al*., 2007). Therefore, an alternative to increase the number of buffalo that are inseminated is the use of protocols that allow the AI without the need of estrus detection, usually called fixed-time AI (FTAI). Follicular wave development can be controlled by treatments with GnRH or estradiol and progestogen/progesterone in combination. Treatment of buffalo with GnRH in combination with prostaglandin F2α ($PGF_{2\alpha}$) 7 d later and a second GnRH 48 h after $PGF_{2\alpha}$ (known as Ovsynch) has resulted in acceptable pregnancy rates after FTAI in cycling buffalo during the breeding season (Baruselli *et al*., 1999, Berber *et al*., 2002). FTAI protocols using progestin devices, estradiol and eCG have resulted in synchronous onset of a new follicular wave, synchronous ovulation and consistent pregnancy rates in anestrous buffalo during the off breeding season. (Baruselli and Carvalho, 2003). The combination of these protocols permits the use of AI throughout the year, obtaining conception and calving even in anestrus buffalo during the off breeding season.

Recent advances in protocols for superovulation in buffalo

Bovine embryo transfer has been applied widely around the world. This technology increases the number of offspring obtained from donors with high genetic value and is used to disseminate desirable genetics around the world. However, buffalo embryo transfer present low efficiency compared to bovine, making difficult the use of this important technique by buffalo farmers (Drost *et al*., 1983; Baruselli, 1994; Zicarelli, 1994). In our trials buffalo present acceptable follicular response during superovulation (10 to 15 follicles ≥ 8 mm), moderate ovulation rate (~ 60%) and CL yield (5-10) but, in contrast, a low embryo recovery rate (20 to 30%) is observed (Baruselli, 2000). Our results provide strong evidence that low embryo recoveries in buffalo may be explained by a failure of oocyte to entry the oviduct after superovulation. It was also observed that buffalo presented, in average, ovulation rates of 62.8%, which is similar to the one found for bovines (Stock *et al*., 1996). This result suggests that the low efficiency of MOET is probably not related to follicular response or to ovulation during superstimulation treatment. The calving of buffaloes produced by *in vitro* embryo production showed that it is possible to obtain an IVF protocol to this species (Neglia *et al*., 2004; Sá Filho *et al*., 2008).

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Current status of germ cell transplantation and testis graft in vertebrates

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Spermatogonial transplantation involves the removal of stem cells from a donor testis and their replacement into a recipient largely deprived or lacking endogenous spermatogenesis, where these transplanted cells grow to form mature fertile sperm with genetic characteristics of the donor. Since spermatogonial transplantation from mouse-to-mouse was first reported by Brinster and colleagues in 1994, many important developments in this fascinating methodology such as interspecies transplants, transplants from cryopreserved and cultured spermatogonial stem cells have been made. In this regard, this technique has been shown a powerful approach to studying the biology of spermatogonial stem cells and their microenvironment, the stem cell niche. Also, several important functional questions regarding Sertoli-germ cell interactions and the role of the Sertoli cell and germ cells during spermatogenesis have now been answered. Transplantation of cultured spermatogonial stem cells is now opening exciting possibilities for *in vitro* multiplication of male germ cells and transfection or retroviral transduction has shown that it is now possible to produce transgenic mice. By freezing and storing testicular tissue, it should be possible to preserve indefinitely the genetic stocks of valuable farm animals, endangered species and unique experimental animals, until a suitable recipient can be found that will maintain the germ line. Also, because many cases of male infertility have proved intractable to therapy, spermatogonial transplantation has also potential clinical applications to address human infertility. For instance, if the testis of infertile individuals contains at least spermatogonial stem cells, it might be possible to transplant these cells into a host testis of the same or different species to obtain sufficient sperm of donor origin to achieve a pregnancy using ICSI. Another potential clinical use of spermatogonial transplantation technique is the replacement of the germ line in patients whose endogenous stem cells had been eliminated (or damaged) as a result of gonadotoxic chemo-or radiotherapeutic treatment.The spermatogonial transplantation efficiency is still relatively poor and presents limitations related to the preparation of donors, recipients, and the quality of germ cell development after transplant. Also, although germ cells from rabbits, dogs, large domestic animals (boar, bull and horse), primate (baboon) and humans into nude mice were able to colonize the testis at different degrees, no spermatogenesis of the donors occurred. These results suggest that the success of transplantation might be positively related to the degree of phylogenetic proximity of species during evolution. To bypass this limitation, recent investigation grafting small pieces of testis tissue from several species into nude mice hosts showed that male germ cells could be propagated and produce fertile spermatozoa. This approach created a totally new scenario in this field allowing, for instance, the production of transgenic domestic animals. However, regardless of its limitation germ cell transplantation has been proved to be an extraordinary and powerful technique to investigate reproductive biology and stem cell biology in mammals and, more recently, in other vertebrates such as fish.

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In vitro culture of caprine preantral follicles enclosed in ovarian tissues: main results from LAMOFOPA-UECE (Fortaleza, Ceará, Brazil)

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The main aim of the researches developed in the Laboratory of Manipulation of Oocytes and Preantral Follicles (LAMOFOPA) is to develop a culture system which ensures high rates of survival, activation and *in vitro* growth of caprine primordial follicles. Such studies are very important to understand the factors that control the early folliculogenesis. The ovaries used in our study were obtained at local slaughterhouse from adult cyclic goats and transported to the laboratory in Minimal Essential Medium (MEM) supplemented with antibiotics. At the laboratory, each pair of ovary was divided into several cortical fragments and fixed in Carnoy (fresh control) or cultured in 1 ml of control medium. This medium was composed of MEM supplemented with Bovine Serum Albumine (BSA), ITS (Insulin, Transferrin and Selenium), Pyruvate, Glutamine and Hypoxantine (MEM^+) or MEM^+ plus different concentrations of one of the following substances: Follicle Stimulating Hormone (FSH), Estradiol, Progesterone, Growth and Differentiation Factor-9 (GDF-9), Insulin like Growth Factor-1 (IGF-1), Growth Hormone, Vasoactive Intestinal Peptide (VIP), Kit Ligand (KL), Bone Morphogenetic Protein-6 (BMP-6), -7 (BMP-7) and -15 (BMP-15), Angiotensin (ANG), Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Fibroblast Growth Factor-2 (FGF-2). The cultures were performed at 39ºC in 5% CO2 in air for 7 days. To evaluate the efficiency of the media, parameters such as the percentage of follicle survival (by histological and transmission electron microscopy analysis), activation of primordial follicles and follicular and oocyte growth were take into consideration. The results showed that after 7 days of culture, some of the factors (FSH, Progesterone, GDF-9, IGF-1, VIP, EGF) maintained preantral follicles survival similar to fresh control. In addition, other factors increase follicular activation (FSH, Estradiol, Progesterone, GDF-9, IGF-1, FGF-2, VIP, BMP-7, EGF) or follicular diameter (FSH, Progesterone, GDF-9, IGF-1, FGF-2, VIP) when compared with fresh control. Furthermore, FSH, Estradiol and GDF-9 were more efficient to promote both primordial follicles activation and an increase in follicular diameter than MEM+ alone. Overall, the results demonstrated that all substances tested are important in the control of early folliculogenesis depending on the concentration used.

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What advances are needed before grafting of cryopreserved ovarian tissues can reach full potential in animal conservation

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Grafting of cryopreserved ovarian tissue commenced over 50 years ago and the first report of mouse pups being born came in 1961. Current cryopreservation and grafting protocols make germline preservation and propagation a useful tool that has resulted in fertility restoration (offspring) in moths, mice, rats, sheep and humans. In mice, the most well studied of all species, a wide range of collection, processing, cryopreservation and grafting strategies (including xenografting), have successfully resulted in normal, healthy offspring.

Although there is considerable interest in, and a need for, cryobanking of ovarian tissue to preserve the female germline for threatened wildlife species, its usefulness is currently moderated by various practical issues, including how to obtain, process and replace and monitor the ovarian tissue. Our interest is in how to optimize the replacement step.
In human medicine, ovarian tissue banking is primarily aimed to help young women who are at risk of early ovarian failure, by storing their tissue and autografting it back to them when they are ready to have children. This approach has limited or no value for animal conservation as there are essentially no instances in which the same individual animal would benefit from having its own ovarian tissue collected, stored and then returned to it.

In small animal models all grafting to another individual of the same species is widely used, but to be highly effective the recipient has to be immunologically compromised and/ or fully histocompatible with the donor to avoid rejection. In the case of threatened wildlife species all grafting is graft rejection and disease transmission are both major problems (Shaw et al., 1996; Gosden, 2007; Wei *et al.,* 2007; Petroianu *et al*., 2007). In our opinion the most valuable advance would therefore be the development of strategies to prevent immune rejection without, aggressive, immunosuppressive drugs, as this would permit allografting. Allografting has several advantages over *in vitro* culture and ensures that follicles and oocytes are grown in a physiologically compatible environment. If allografting became feasible then ovarian tissue from a genetically valuable individual could be placed in several different (less valuable) recipients and thereby greatly boost the donors reproductive potential; comparable to propagating the male germline via artificial insemination. Tissue collection from a living endangered animal is problematical, but an advantage of ovarian tissue is that functional material is recoverable even for a few hours after death.

Another approach that has received attention is the use of xenotransplantation (the transplantation from one species to another) of ovarian tissue to an immunologically compromised recipient; to date rodents have been the most commonly used recipients. This approach can support antral follicle development in a wide range of species including marmoset, elephant, hamster, dog, wallaby, wombat, cat, macaque, cow, pig, rabbit, goat (Paris *et al.,* 2004). The current practice of using immunocompromised mice and rats as xenografting recipients does have limitations including a) the small size of the recipient animals and b) the physiological discrepancies between the donor and recipient which can have consequences for the development of healthy, fertilizable oocytes. On a positive note, live offspring has been achieved in a xenogeneic setting (mouse to rat), but further work is needed to determine the potential of this approach for grafting ovarian tissue over greater xenogeneic barriers.

Thus, in summary our presentation will focus on the identification of research needs to address whether and how the tools of ovarian cryopreservation combined with ovarian grafting can aid to the propagation of threatened wildlife species.

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Human luteolysis

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Objectives
To discuss luteolysis during the human menstrual cycle and pregnancy.

Materials and Methods
Studies to evaluate the regression of the corpus luteum (CL) during the human menstrual cycle and pregnancy using histology, ultrasonography, and molecular and cellular techniques are discussed.

Results
Luteolysis occurs approximately 7 days after ovulation in the absence of conception (1, 10). The corpus albicans can be detected, but is not functional, during the follicular phase of the menstrual cycle (1). Following conception, the syncytiotrophoblast cells of the developing conceptus secrete human chorionic gonadotropin which maintains the CL until approximately 20 weeks gestation, at which time the luteal-placental shift has occurred and the CL regresses (8). The signal for luteolysis in women is not well known; however, it is thought that the signal may involve a loss of gonadotropic support (9). Recent evidence indicates that both apoptotic (12, 14) and authophagic (6) processes are involved in luteal regression. Luteolysis is characterized by decreasing size and number of luteal cells (11), decreasing vascularity (13), tissue remodeling (4), and infiltration of lymphocytes and macrophages (7). Luteal diameter and area decrease during luteolysis (1,10), in association with a decline in serum progesterone and estradiol concentrations (1). In addition to endocrine hormones, growth factors (2,16), cytokines (3), reactive oxygen species (15) and nitric oxide (5) appear to modulate luteal regression in women. The timing of the onset of luteal regression, peak progesterone concentrations and peak estradiol concentrations have not yet been shown to differ in women with 2 versus 3 follicular waves during the menstrual cycle (1).

Conclusions
Luteolysis is a complex phenomenon, regulated by a precise network of endocrine, autocrine, and paracrine factors. Morphologic changes in the CL during the human menstrual cycle and pregnancy have been well-documented, due to marked improvements in ovarian imaging. Significant advances in cellular and molecular techniques are providing new insight into the biologic mechanisms underlying luteolysis, which will assist in the treatment of women experiencing luteal deficiency.

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Ovarian toxicity in the adult rats treated with anabolic androgenic steroids: histological evaluation and follicular quantitation

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Introduction

The abusive and indiscriminate use of androgenic anabolic steroids (AAS) is an usual practice among men and women, athletes or not, searching for short-time increases in muscular mass (1). Some studies showed the deleterious effects promoted by AAS in animals, mainly rodents and stallions. The AAS are used therapeutically for various disorders, however, promote side effects which include, reduced fertility, hepatic neoplasms and carcinoma, tendom damage, blood clotting and behavioral disorders (2). In the females inhibition of folliculogenesis and ovulation (3), and alterations on the estrous cycle (4), are described. It is well documented in the literature the effects of AAS on the male reproduction, but little is known about the effects from these drugs in the females. Here, the goal of this study was to analyze the effects of single or combined use of two commercial AAS in the histological structure and follicular population of ovaries of adult rats.

Materials and Methods

Twenty Wistar rats were casually distributed in four experimental groups (n = 5/group): a) control (C- physiological solution); b) treated with nandrolone decanoate (ND- 7.5 mg/kg BW); c) treated with compounds of testosterone (T- decanoate, propionate, fenilpropionate and isocaproate; 7.5 mg/kg BW); d) treated with ND + T (7.5 mg/kg BW of each steroid). The treatments were given in a single dose/week, intraperitoneal, during 8 consecutive weeks. The estrous cycle was monitored daily. Serial sections of ovaries, stained with hematoxylin-eosin, were analyzed by light microscopy for evaluation of gonadal tissue and the counting of follicles was performed in both ovaries, according to the classification proposed by Pedersen and Peters (5).

Results and Discussion

The females treated with steroids presented higher ($p < 0.05$) body weight, estral acyclicity, lack of follicular units and absence of corpora lutea in the ovaries, confirming previous studies (3,4). In the group treated simultaneously with ND/T, there was also a decrease ($p < 0.05$) in the gonadal weight and increase in the quantity of atretic follicles, in comparison to control group. The association of ND and T promoted toxicity in the ovarian tissue of adult female rats, reinforcing the deleterious effects promoted by isolated use of each steroid.

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Evaluation of the ovary in rats after *in utero* and lactational exposure to low dose of phthalate

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Introduction

Phthalate esters are used as plasticizers to impart flexibility to polyvinyl chloride (PVC). They are also used in many products, like medical devices (IV storage bags, ventilator tubing, catheters, blood storage and gloves, flexible plastics (children's toys, bottles and feed bottles), some personal care products and thus have ubiquitous environmental presence. Some studies have shown that *in utero* and lactational exposure to high doses of phthalates (500 mg /kg / day to 2g /kg / day) may cause important reproductive disorders (Ema et al., 1993, 1994; Gray et al., 2006; Hirosawa et al., 2006; Grande et al., 2006) since they have the potential to bind to estrogen receptors, thus acting as endocrine disruptors. However, there is a lack of studies on lower doses (close to environmental exposure). Based on that, this study aimed to evaluate the possible effects on the ovaries, in rats whose mothers were exposed to a low dose of DBP (Di-η-butyl phthalate) during pregnancy and lactation.

Materials and Methods

Pregnant rats were divided into two experimental groups: treated (T, n = 10) received DBP, gavage, by intragastric administration, 100 mg/kg/day, from gestational day (GD) 12 until postnatal day (PND) 21, which is the critical period for reproductive system development of offspring; the control group (C, n=9) received corn oil (vehicle), under the same experimental conditions. To assess the fetal ovaries, C (n = 5) and T (n = 5) pregnant dams were killed by decapitation on GD20 for fetal gonad collection and analysis. Germ cells were counted in ovarian sections and expressed as number of cells per unit area (nº germ cells/mm^2).

At PND 60, ovaries were collected and weighed from control and treated rats in estrous phase. Ovarian follicles (primordial/primary, pre-antral, antral and atretic) and corpora lutea were counted. For comparison of results between the experimental groups, "T" of Student test was utilized. Differences were considered significant when $p < 0.05$.

Results and Discussion

In the fetal ovaries, the number of germ cells per unit area (mean ± SEM, C= 3402.05 ± 218.41; T = 2847.01 ± 215.13) was comparable between groups. Similarly, the ovary weights (mg) at PND60 (C = 98.00 + 13.78; T = 93.60 + 6.59), as well as the numbers of ovarian follicles and corpora lutea, were unaffected by the treatment. According to these results, we suggest that exposure to DBP, in these experimental conditions, did not provoke toxic effects on the ovary of rats.

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Nellore heifers estrous cycle during different seasons of the year

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Introduction

Recent research found seasonal variation on the mount frequency of beef cattle (1) and the estrous length and ovulatory interval, although there was not seasonal influence on small follicles population (3-6mm) of Guzerá (2). There is seasonal variation of LH secretion in intact Nellore heifers but it is less evident in ovariectomized ones. The variation of negative feedback of estradiol may be important during seasonal LH variation (3). This research evaluated physiological variation on follicular population in Nellore heifers in different seasons of the year.

Material and Methods

There were used eight Nellore heifers (*Bos indicus*) with 4 years of age, weighted every 30 days, followed for two estrous cycles per season. Before each cycle evaluation, animals were synchronized with two administration of Cloprostenol (0.530 mg, IM) 12 days apart. After 72 h from the second administration ultra-sonographic (GE Logic 100, 5 MHz probe) exams were conducted daily until ovulation, follicles larger than 3 mm were measured and counted. Data were analyzed by repeated measure Anova (SAS). Photoperiod data was obtained from apolo11.com website.

Results and Discussion

The data presented on Table 1. Body weight decreased in Autumn and Winter while and number of follicles decreased in Summer and Autumn. The ovarian follicular population did not follow the weigh variation. Although nutritional status is important for reproduction, it did not explain the seasonal variation on follicular population. The number of follicles in Summer and Autumn followed the reduction on the photoperiod. Plasmatic LH may explain this variation. It will be necessary its quantification during these periods to evaluate its importance on these follicle variations.

Table 1. Body weigh, follicle number and preovulatory follicle diameter (average ± SEM) evaluated in two estrous cycle in each season of the year using eight Nellore heifers (n = 8), and the change on the photoperiod

Parameters	2007			2008
	Autumn	Winter	Spring	Summer
Body weight (kg)	570.3 ± 15.6^{a}	586.0 ± 15.5^{a}	655.6 ± 19.3^{b}	625.9±17.9^{b}
Follicles total	8.5 ± 0.2^{b}	9.7 ± 0.2^{a}	10.9 ± 0.2^{a}	7.8 ± 0.2^{b}
3mm ≤ Follicles ≤ 5mm	6.9 ± 0.2^{bc}	8.0 ± 0.2^{ab}	8.9 ± 0.2^{a}	6.1 ± 0.2^{c}
5mm < Follicles ≤ 9mm	1. 0 ± 0.1^{b}	1.2 ± 0.1^{ab}	1.4 ± 0.1^{a}	1.2 ± 0.1^{ab}
9mm < Follicles ≤ 14mm	†0.5^{a}	†0.5^{a}	†0.5^{a}	†0.5^{a}
Follicles > 14mm	§†^{a}	§†^{ab}	§†^{b}	§†^{ab}
Preovulatory follicle diameter (mm)	12.1 ± 0.5^{a}	12.2 ± 0.4^{ab}	12.3 ± 0.4^{b}	13.0 ± 0.5^{ab}
Change on the photoperiod (hours:minutes)	-1:16	1:15	1:19	-1:18

Different superscript letters show different line values (p < 0, 05) Tukey test.
§Average was lower than 0.05.
†Standard error was lower than 0.05.

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Dominant follicle growth is interrupted by intrafollicular injection of FGF10 in cattle

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Introduction

Fibroblast growth factor 10 (FGF10) is expressed by oocytes and theca cells from bovine antral follicles, and its expression is negatively correlated with estradiol levels in the follicular fluid (1). FGF10 receptor (FGFR2IIIB) is expressed by mural and cumulus granulosa cells (1,2) and corpus luteum (3). When added to granulosa cells culture, FGF10 decreases estradiol secretion (1) and expression of angiotensin II receptor type 2 (AT2; 4). Thus, the aim of this study was to test the hypothesis that FGF10 acts as an inhibitor of dominant follicle development around deviation in cattle.

Materials and Methods

Eighteen cows were submitted to a hormonal protocol in order to induce a new follicular wave. Ovaries were daily monitored and when the largest follicle reached the diameter of 7-8 mm, it was injected PBS (Control group; n = 3) or human recombinant FGF10 at 100 ng/mL (FGF100 group; n = 4) or at 1,000ng/mL (FGF1,000 group; n = 3). The injected amount was calculated based on the volume of follicular fluid estimated by a linear regression equation (5), to obtain an adequate final concentration inside the follicle. After the injections, animals were daily monitored by ultrasound during three days to evaluate effects on the follicular dynamics.

Results and Discussion

The intrafollicular injection of FGF10 at 100 or 1,000ng/mL interrupted follicular growth compared to control group (Fig. 1). FGF10 was shown to be downregulated in theca cells of healthy steroidogenic follicles and to decrease estradiol production of cultured granulosa cells without affecting cell proliferation (1). Recently, our group demonstrated that angiotensin II signaling is necessary for the continuation of follicular growth near follicle deviation (6), and that the expression of AT2 receptors is inhibited by FGF10 in cultured granulosa cells (4). Taken together, these results suggest that FGF10 acts as an important regulator of follicular growth in cattle. We propose that FGF10 expression needs to be suppressed in order to allow the continuation of follicle growth after deviation.

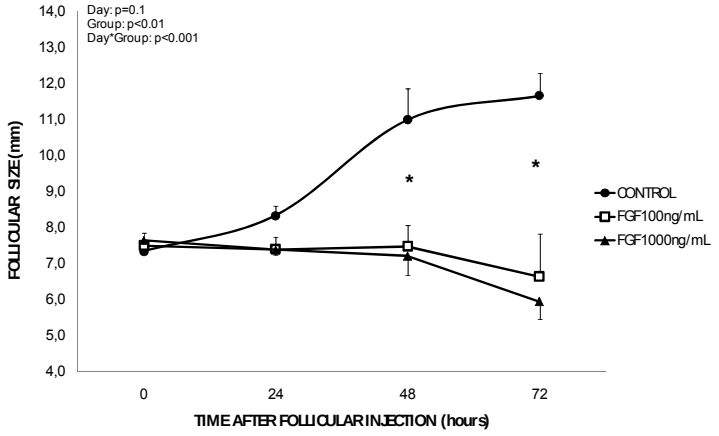

Figure 1. Follicular dynamics observed after injection of PBS (control) or FGF10 in doses of 100 or 1,000ng/mL. *indicate statistical significance.

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Expression of fibroblast growth factor homologous factors (FHFs) in bovine antral follicles

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Introduction

Several members of the fibroblast growth factor (FGF) family are involved in the regulation of ovarian function. A small FGF subfamily encodes non-secreted proteins otherwise known as FGF homologous factors (FHF) that are principally expressed in the developing and mature nervous system, where they bind to MAPK scaffold proteins and to voltage-gated sodium channels (1). Expression of FHFs has not been investigated in reproductive tissues. The aims of this study were to assess FHF (1-4) mRNA expression in granulosa (GC) and theca cells (TC) from bovine antral follicles at different developmental stages, to determine if expression in GC is regulated by FSH and IGF-1, and to localize FHF-2 protein in the bovine antral follicle.

Materials and Methods

Bovine ovaries were obtained at a local abattoir, TC and GC cells were dissected from individual antral follicles larger than 4 mm and submitted to total RNA extraction. Follicles were grouped according to size (small: 5-7 mm; medium: 8-10 mm; large: >10 mm) and health status based on the ratio between concentrations of estradiol and progesterone measured by RIA in the follicular fluid (healthy: >1; transitional: 0.01-1 and atretic: <0.01). To investigate regulation of FHFs mRNA by FSH and IGF-1, GC from small follicles (2-5 mm) were placed into serum-free medium supplemented with insulin and bovine FSH (0, 0.1, 1, 10 or 100 ng/ml) or IGF-1 (0, 5, 10, 50 or 100 ng/ml). FHFs gene expression was examined by semiquantitative real time RT-PCR using bovine-specific primers and GAPDH, cyclophilin A and histone H2a as endogenous controls for GC, TC and cultured GC, respectively. Effects of follicle status, size and treatment on FHF expression were tested by ANOVA and groups were compared by Tukey-Kramer HSD test ($p < 0, 05$; JMP software). Immunohistochemical analysis was performed with a polyclonal human FHF-2 antibody (Abnova; 1:200).

Results and Discussion

FHF-1 mRNA was detected without changes in TC, but increased with health status and size in GC. Culture data revealed that FHF-1 expression is upregulated by FSH but not by IGF-1. FHF-2 mRNA expression decreased with health status in both GC and TC, where it was highest in large atretic follicles, and was not significantly altered by FSH or IGF-1 in cultured GC. In accordance with mRNA data, FHF-2 protein was localized to both TC and GC. In TC, FHF-3 mRNA expression decreased with health status in medium follicles and increased with size in healthy follicles, whereas it increased with size in GC. FSH had a biphasic effect on FHF-3 expression in cultured GC, which was lower at the 0.1 ng/ml dose compared with the 10 and 100 ng/mL doses. Lastly, FHF-4 mRNA expression did not vary in TC, but decreased with health status in GC. IGF-1 but not FSH decreased FHF-4 mRNA levels in cultured GC. In conclusion, the expression patterns differ between FHFs during antral follicle development. Whilst FHF-1 is upregulated in healthier and larger follicles, FHF-2 and FHF-4 are enhanced in atretic follicles. Moreover, the controlling mechanisms of mRNA expression also seem to vary between FHFs in GC.

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Renin-angiotensin system (RAS) components are overexpressed after PMSG treatment in the rat ovary

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Introduction

Many studies point to an involvement of the ovarian Renin-Angiotensin System (RAS) as a paracrine/autocrine regulator of follicular development, steroidogenesis, oocyte maturation, ovulation, and follicular atresia [1]. In spite of these studies, the role of Angiotensin II (Ang II) in the ovarian physiology was not completely clarified. Furthermore, Angiotensin-(1-7) [Ang-(1-7)], an new active peptide of the RAS has been identified in the ovary [3]. Ang-(1-7) can be generated from Ang I by neutral-endopeptidase (NEP) and prolil-endopeptidase (PEP) or from Ang II by PEP or angiotensin-converting enzyme 2 (ACE-2) [2]. Costa *et al*. (2003) showed that Ang-(1-7) induces estradiol and progesterone production by in vitro perfused rat ovaries, an effect blocked by A-779, a specific Ang-(1-7) antagonist. More recently, it has been demonstrated that Mas protein is a specific receptor for Ang-(1-7) [4]. The present study was undertaken to identify the cellular localization of Ang-(1-7) and the expression of Mas receptor and ECA2 in the rat ovary following gonadotropin stimulation.

Materials and Methods

1) Animals: female Wistar immature rats (23-25 days old) were injected *sc* with PMSG (20 IU) or vehicle solution and sacrificed 48 hours later.

2) Immunohistochemistry: after anesthesia and laparotomy, rats were perfused with 4% paraformaldheyde, ovaries were removed, embedded in paraffin, sectioned at 4 μm and mounted on gelatinized slides. Tissue distribution of Ang-(1-7), Ang II and Mas receptor was evaluated by immunohistochemistry, following the avidin-biotin-peroxidase method, using rabbit polyclonal specific antibodies. The immunostaining was visualized with DAB and counterstained with hematoxylin.

3) Real Time Polimerase Chain Reaction: mRNA expression levels of Mas receptor and ECA2 were evaluated by quantitative RT-PCR. Rats were sacrificed by decapitation and ovaries immediately removed, frozen in liquid nitrogen and stored at -80^{o}C for RNA extraction according phenol-guanidine isothiocyanate (Trizol) protocol. cDNA was synthetized from 1 μg total RNA and subsequently submmited to real time PCR analysis, conducted following manufactor's procedures (Apllied Biosystems) using SYBR Green Master Mix. Specific primers for Mas and ECA2 were used and expression levels were normalized to the S26 housekeeping gene and calculated as $2^{-\Delta\Delta Ct}$.

Results and Discussion

PMSG treatment increased Ang-(1-7), Ang II and Mas immunoreactivity in rat ovaries: strong staining for Ang-(1-7) and Mas was observed mainly in thecal-interstitial cells, while Ang II was more evident in the granulosa layer. The expression of Mas mRNA was higher in treated (3.36 ± 0.48 UA) compared to control animals (1.66 ± 0.47 UA). Also, PMSG treatment enhanced ECA2 mRNA levels (2.15 ± 0.26 UA) compared to control (1.29 ± 0.25 UA), suggesting that this enzyme can be responsible for Ang-(1-7) synthesis in the gonadotropin-stimulated rat ovary. These data provide evidence for gonadotropin-induced changes in the ovarian expression of renin-angiotensin system components. Hence, we may suggest that the Ang-(1-7)/Mas axis, present in theca-interstitial cells, could stimulate the production of androgen precursors, which are later converted to estradiol in granulosa cells under Ang II action.

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Immunolocalization of BRG1 – SWI/SNF protein during folliculogenesis in the porcine ovary

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Introduction

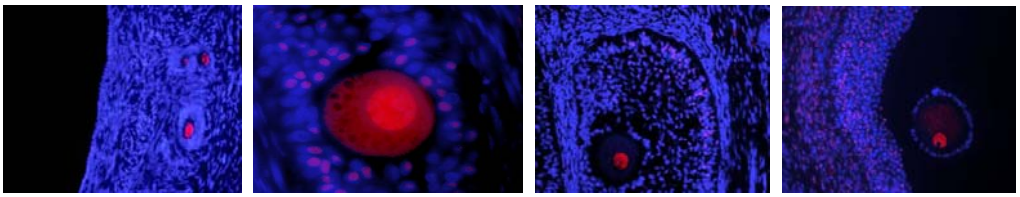
Dynamic changes in chromatin structure and gene expression occur during follicular and oocyte growth. Epigenetic mechanisms regulate these changes through biochemical reactions that modify the nucleosome structure, affecting DNA expression. Chromatin remodelers that alter DNA-histones interactions can influence transcriptional activity by facilitating or repressing DNA access. The SWI/SNF complex represents an important chromatin remodelling family, which comprises many proteins subunits including the BRG1 (brahma-related gene 1). Our aim in this study was to perform a qualitative approach of BRG1 expression patterns in different stages of follicular development.

Material and Methods

Ovaries (n = 10) were collected from pre-pubertal gilts after evisceration and then rinsed in cold PBS. Ovarian fragments were cut and placed into a 4% paraformaldehyde solution at 4˚C for 12 hours. Tissues were processed using standard histological protocols. For immunofluorescence, samples were deparaffinized and rehydrated through three changes of alcohol. After washing in PBS-Brij, antigen retrieval was performed by incubating the tissue sections in sodium citrate buffer for 5 min in a pressure cooker. Sections were subsequently blocked for 1 h with normal goat serum and then incubated with primary antibodies, polyclonal rabbit anti-BRG1 (1/200) or control rabbit IgG at the same concentration, overnight at 4˚C. Primary antibodies were detected using Alexa Fluor 594-anti-rabbit 1/1000 diluted secondary antibody. Cells were counterstained with DAPI. We evaluated primary, secondary, tertiary and antral follicles.

Results and Discussion

Positive fluorescent signal for BRG1 was detected in all analyzed samples. In primary follicles, only oocytes showed BRG1. Secondary follicles expressed positive signals in the oocyte and few granulosa cells. At the tertiary stage, a strong expression was identified in the oocyte, suggesting a co-localization with euchromatin. At this stage, only granulosa cells close to theca layer were positive. On antral follicles, almost all granulosa and theca cells showed positive signals to BRG1, suggesting a possible role on proliferative and steroidogenic events. Our results suggest an important role of BRG1 in all steps of oocyte development, since primary to antral phase. Granulosa and theca cells showed positive signals especially at steroidogenic steps. Considering BRG1 a candidate gene for embryonic genome activation (1), our results suggest an important role of this chromatin remodeler since oocyte into primary follicles. The strong signal during steroidogenic phases is in accordance with BRG1 activity in estrogenic receptors (2). Finally, we consider the possibility of an important role played by BRG1 during growth and differentiation of oocytes and follicles, possibly playing activities at cellular proliferation.



Primary follicle Secondary follicle Tertiary follicle Antral follicle

Figure 1. Immunofluorescence of BRG1 expression (red) in primary, secondary, tertiary and antral follicles (blue, DAPI stain).O oocyte, GC granulosa cells, TC theca cells.

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Messenger RNA quantification of bone morphogenetic protein-6 (BMP-6) in goat ovarian follicles

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Introduction

The identification of the biological functions of autocrine/paracrine factors that are produced in the ovary is very important to understand the mechanisms controlling folliculogenesis. Some of these molecules are synthesized and secreted by the oocyte and/or granulosa cells and control follicle growth and differentiation. Bone morphogenetic protein-6 (BMP-6) has been identified in rat ovarian follicles and some evidences indicate that it controls granulosa cells activity (Shimasaki et al., 2004). However, in goats, relatively little information is available on the local factors that regulate folliculogenesis. The aim of the present study was to perform the quantification of mRNA for BMP-6 in goat ovarian follicles.

Material and Methods

Ovaries (n = 30) of goats were collected in local slaughterhouses. Ten ovaries were dedicated to isolate primordial, primary and secondary follicles, using a mechanical tissue chopper. The remaining ovaries were used to collect COCs and mural granulosa / theca cells after dissection of small antral follicles (<3 mm) and large antral follicles (3-6 mm). Groups of ten primordial, primary or secondary follicles as well as ten COCs and samples of granulosa / theca cells from small and large antral follicles were collected and stored at –80oC until RNA extraction. Quantification of mRNA for BMP-6 was performed by real time PCR, using a solution containing SYBR green and specific primers. GAPDH and β-actin were used as endogenous controls. Data of mRNA expression among primordial, primary and secondary follicles were analysed by ANOVA and means were compared by Tukey test, while t test was used for paired comparisons of mRNA expression in small and large antral follicles ($P < 0.05$).

Results and Discussion

Quantification of mRNA expression demonstrated a significant increase in the production of BMP-6 during the transition from primordial to primary and secondary follicle stages ($P < 0.05$). However, no significant difference in the expression of BMP-6 was observed between primary and secondary follicles. With regard to antral follicles, a reduction in the synthesis of mRNA for BMP-6 was observed in both COCs and granulosa / theca cells from large antral follicles when compared with small antral follicles, but the difference were not significant ($P > 0.05$). In rats, BMP-6 mRNA expression is rapidly decreased at the time the dominant follicle is selected and may be linked to the mechanisms of follicle selection. In vivo studies have demonstrated that BMP-6 inhibits progesterone production induced by FSH (Otsuka et al., 2001). When the expression of BMP-6 was compared between COCs and granulosa / theca cells of small or large antral follicles, no significant difference was observed ($P > 0.05$). In rats, BMP-6 is also expressed in both oocyte and granulosa cells (Shimasaki et al., 2004). In goats, the protein for BMP-6 has been localized in oocyte and granulosa cells of both preantral and antral follicles (Silva et al., 2007). It is concluded that, an increase in the production of BMP-6 is observed during transition of primordial to primary follicles in goats and that both COCs and granulose/theca cells from antral follicles produce similar levels of BMP-6 mRNA.

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Repeatability of follicle diameters and hormones in mares

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Introduction
Measurable repeatability within an animal has been shown in association with number of follicles and superovulation rates in cattle and sheep. In humans, the number of antral follicles had a measurable degree of repeatability in individuals between menstrual cycles. The few repeatability studies performed in these species have been concentrated mainly in follicle numbers. Mares are good comparative research models for follicle studies because of many similarities with women in the dynamics of the ovulatory wave. In mares, repeatability has not been studied previously for any reproductive characteristic. The purpose of the present studies was to characterize the within-mare repeatability between two consecutive intervals for follicle, FSH, LH, and estradiol dynamics in spontaneous (experiment 1) and ablation-induced (experiment 2) follicle waves.

Materials and Methods
Transrectal ultrasonographic examination of the ovaries and blood sampling for hormone assays were performed daily. Numbers of follicles were grouped into diameter classes in each experiment. In experiment 1 (n=23 mares), the experimental period extended from 4 days before the first ovulation to 4 days after the third ovulation (ovulation = Day 0). Diameters of follicles ≥15 mm were measured and diameters of all other follicles (2 to <15 mm) were estimated using a calibrated grid. In experiment 2 (n=24 mares), an ovulatory wave was induced 10 days after ovulation by ablation of all follicles ≥6 mm by transvaginal ultrasound-guided aspiration and injection of 5 mg of PGF2α. The same procedure was repeated in the next cycle. After ablation, the identity of follicles ≥6 mm of the induced ovulatory wave was maintained. Correlations between the two interovulatory intervals (IOIs) or between the first and second postablation periods as an indication of repeatability for a specific day and end point were carried out by the Spearman test.

Results and Discussion
In experiment 1, repeatability within mares was based on the following positive and significant correlations between the two IOIs: 1) length of the interval between ovulations ($r = +0.67$, $P<0.0005$) and between ovulation and the beginning of follicle deviation ($r = +0.58$, $P<0.004$); 2) diameter of the preovulatory follicle on Days -3 to -1 ($r = +0.44$ to 0.56, $P<0.03$); 3) number of follicles in diameter classes of 2–5 mm (significant correlation for 22/23 days of the IOI), 5.1–10 mm (18/23 days), 10.1–15 mm (12/23 days), and 15.1–20 mm (12/23 days); and 4) concentrations of FSH (18/23 days, $r = +0.55$, $P<0.04$) and LH (22/23 days, $r = +0.59$, $P<0.04$). The greatest repeatability for the number of follicles within classes occurred in the 2–5 mm class, and thereafter the repeatability progressively decreased as the diameters for the classes increased ($r = +0.40$ to 0.65, $P<0.05$ to $P<0.01$). There was no significant correlation in estradiol concentrations for days centered on the estimated beginning of follicle deviation. The growth rates of the preovulatory follicle were not correlated between IOIs within mares. In experiment 2, results demonstrated measurable repeatability within mares for several end points before, during, and after expected follicle deviation, including the number of follicles 6–9 mm ($r = +0.53$, $P<0.02$) and 10–14 mm ($r = +0.44$, $P<0.05$) during the common-growth phase. Stronger and significant correlations were observed for diameter of the preovulatory follicle at maximum ($r = +0.70$, $P<0.01$) and on the Day -1 ($r = +0.66$, $P<0.02$) for waves with only one ovulation. Concentrations of FSH and LH each showed a significant and positive correlation ($r = +0.44$ to $+0.84$) on the days of ablation, emergence, deviation, ovulation, at minimal value, and at maximal value. Evaluation of data for both experiments did not suggest that repeatability was confounded by age or month. The high repeatability for number of follicles recruited in the wave may have applied implication on the selection of mares more responsive to superovulation treatments. The finding that the preovulatory follicle tends to reach a diameter that is characteristic of the mare may be useful knowledge in equine breeding programs. The significant positive correlations for diameter of the preovulatory follicle occurred on the three days before ovulation. These observations suggest that knowledge of the mare's history on the follicle diameter preceding ovulation may be useful for estimating the optimal diameter for a given mare for ovulation induction, as well as for the optimal time for breeding before spontaneous ovulation. The reliability and practicality for the use of these findings would require a specific study. The present results in mares may lead to investigations in women.

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Age effects on follicle and hormone dynamics in mares

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Introduction
The striking similarities between mares and women in follicle dynamics and hormonal changes during the interovulatory interval (IOI; 1,2) have provided encouragement for the potential use of the mare as a relevant experimental model for the study of folliculogenesis and reproductive aging in women (3). The objectives of the present studies during the estrous cycle in mares were to characterize and compare age groups for follicle development; systemic concentrations of FSH, LH, estradiol, and progesterone; concentrations of preovulatory follicular-fluid hormones and factors; B-mode and color-Doppler characteristics of the follicle wall; and preovulatory oocyte characteristics.

Materials and Methods
Young (5 to 6 yr), intermediate (10 to 14 yr), and old (≥18 yr) age groups of mares separated by 4 yr were used (n = 14 to 16 IOIs/group) in experiments 1 and 3. In experiment 2, the age groups were continuous and were defined as young (3 to 7 yr, n = 11), intermediate (8 to 17 yr, n = 23), and old (≥18 yr, n = 10). None of the old mares were senescent or approaching senescence (4). In experiment 1, transrectal B-mode ultrasonographic examinations and blood sampling were done daily to encompass two consecutive IOIs. In experiment 2, mares were treated with 2500 iu of hCG when the largest follicle was ≥32 mm (Hour 0), and follicular fluid and the oocyte were collected at Hour 30 by transvaginal ultrasound-guided aspiration. Only mares with undetected plasma hCG antibodies at Hour 0 were used. At Hours 0 and 30, diameter of the follicle was measured, blood samples were collected, and the follicle wall was examined by Doppler ultrasonography (5). In Experiment 3, the induction of an ovulatory wave with prostaglandin F2α treatment and ablation of follicles ≥6 mm on Day 10 was used and daily ultrasonographic examinations and blood collection were performed.

Results and Discussion
In experiment 1, the IOI was one-day longer ($P < 0.05$) in the old group in association with a slower ($P < 0.05$) growth rate of the ovulatory follicle. The old group had diminished follicle activity, as indicated by significantly ($P < 0.05$) smaller and fewer follicles. Concentrations of FSH did not differ among age groups, except that the maximum concentration was greater ($P < 0.05$) in the old group. Concentrations of LH were greater ($P < 0.0001$) in the young group throughout the ovulatory LH surge and may have played a role in a shorter ($P < 0.05$) interval from maximum diameter of the preovulatory follicle to ovulation. Maximum circulating concentration of estradiol during the preovulatory surge was greatest ($P < 0.05$) in the young group. In experiment 2, no age effect was detected on oocyte status (meiotic stage, spindle orientation and quality, and microfilament content). Concentrations of ovarian steroids in the preovulatory follicular fluid were not affected by mare age, but the concentration of free IGF1 was greater ($P < 0.05$) in the old group. In experiment 3, during the common-growth phase (Days 12 to 17) of induced waves, diameter of the future ovulatory follicle was not different among ages, but the young group had more ($P < 0.05$) follicles that reached ≥10 mm. Concentrations of LH increased in all age groups during Days 12 to 17, but were greatest ($P < 0.002$) in the young group and continued to be greater ($P < 0.0001$) throughout the ovulatory LH surge. During several days before Day –1, there were no age-related effects on systemic estradiol concentrations, diameter of the preovulatory follicle, or B-mode echotexture or color-Doppler signals of blood flow in the follicle wall. The present studies indicate the importance of the mare as a comparative research model for age effects, for consideration of age as a potential confounding factor in equine research protocols, and for consideration of age in development of theriogenology programs (e.g., optimal time of breeding and superovulation regimes).

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Ovarian follicular dynamics and plasma progesterone concentration and blood metabolites in Alpine breed goats, fed urea in the diet

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Introduction

Ultrasonographic examination of goats ovaries showed estrous cycles with an average of four follicular waves, ranging from two to six (1). Increasing the percentage of ruminal degradable protein in the diet from 11.1 to 15.7 % reduced the number of ovarian follicles of Holstein cows in the post-partum (2). The ammonia or urea excess can alter the hypothalamic-hypophyseal-gonad axis, decreasing the amplitude and the frequency of luteinizing hormone pulses, with the consequent reduction of progesterone (P_4) secretion and, ultimately, the fertility (3). The objective was to study follicular dynamics in goats fed diets containing increasing concentrations of urea.

Materials and Methods

Twenty nine goats were randomly assigned to four treatments that received 0.0% (T1 - Controls, n = 7), 0.73% (T2, n = 7), 1.46% (T3, n = 7) and 2.24% of urea in the dry matter of the diet (T4, n = 8). The ovarian activity was monitored daily by transrectal ultrasonography during one, two or three consecutive interovulatory periods (1). Colorimetric enzyme assays were used to measure concentrations of urea and glucose in blood samples of all goats collected weekly. Blood plasma P_4 concentrations were measured by solid phase radioimmunoassay (RIA) in samples collected on days 3, 7, 11, 15 and 19 after the estrus in goats that showed cycles of normal length.

Results and Discussion

From the 47 estrous cycles evaluated, 59.57, 14.89 and 25.53% were of normal, short and long length (17 to 25; <17 and >25 days), respectively. The normal length estrous cycles had two (n=6), three (n = 13), four (n = 8) and five (n=1) follicular waves. The short and the long length had one and two to 9 follicular waves, respectively. The animals plasma urea and glucose concentration did not differ among the treatments or weeks of collection. A quadratic effect of urea concentration in the diets on plasma P_4 concentration at estrus day ($\hat{Y}$ = 0.07+0.19U-0.07*U^2, r^2 = 0.96, P < 0.05) and on day 11 after estrus ($\hat{Y}$ = 7.16+2.93U-1.54**U^2, r^2=0.99, P < 0.01) was observed. The highest plasma P_4 concentration occurred with the intake of 1.36 and 0.95% of urea in the diets, respectively. At estrus and 11 days after**,** the addition of urea to the diet higher than ones used reduced the plasma P_4 concentrations. On day 15 after estrus, the plasma P_4 concentration was negatively and linearly associated with the proportion of urea in the diets ($\hat{Y}$ = 8.29-0.98*U, r^2 = 0.58, P < 0.05). It is concluded that the interestrus and interovulatory periods, the luteal and follicular phase length and the ovarian follicular dynamics of non-lactating Alpine goats were not affected by the urea concentration up to 2.24% in the diet, but did affect the plasma progesterone concentrations.

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Apoptosis and autophagy are required during the follicular atresia in teleost ovary

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Introduction

Follicular atresia is a hormonally controlled degenerative process by which vertebrate ovarian follicles lose their integrity and are eliminated prior to ovulation. Apoptosis or type I cell death is a well-characterized pathway to cell death essential to maintenance of tissue homeostasis in multicellular organisms. Autophagic or type II cell death has been considered as an alternative route of programmed cell death, distinct from apoptosis and characterized by the formation of many large autophagic vacuoles (1). The purpose of the present study was to investigate the contribution of apoptosis and autophagy during follicular atresia in two freshwater teleost species from the São Francisco River basin, Brazil: curimatã-pacu, *Prochilodus argenteus* and piau-jejo, *Leporinus taeniatus*.

Material and Methods

Fishes were maintained in captivity after the reproductive period and follicular atresia was assessed for three stages: early, advanced, and late atresia. The morphological features of the atretic follicles were histologically examined in ovarian samples fixed in Bouin's fluid for 8 h at room temperature, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. For the identification of autophagy in the follicular cells, the ovarian samples were fixed in Karnovsky, post-fixed in 1% osmium tetroxide with 1.5% potassium ferrocyanide for 2 h, and then embedded in Epon/Araldite plastic resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a transmission electron microscope. The TUNEL *in situ* technique was used to assess apoptosis during the follicular atresia in ovaries of the *P. argenteus* and *L. taeniatus*.

Results and Discussion

The atretic follicles exhibited zona pellucida breakdown, yolk degeneration, and hypertrophied follicular cells, common features of the follicular atresia in most of the teleosts (2). Morphometric analyses of the ovarian regression showed decreased atretic vitellogenic follicles and increased perinucleolar follicles for the future reproductive cycles and yellow bodies to both species. In contrast to mammals, oogonia continue to proliferate in adult females and produce perinucleolar follicles, which stock oocytes for the next reproductive cycle (3). Intense heterophagy to engulf the yolk, and autophagy were detected in the follicular cells during advanced and late atresia. The TUNEL assay detected DNA fragmentation, mainly in late follicular atresia. Differently, the apoptosis contribute greatly for the rapid elimination of the postovulatory follicles in spawned ovaries of *P. argenteus* (4). We inferred that heterophagy, autophagy, and apoptosis contributed to follicular atresia in teleost ovaries, thereby achieving a more efficient removal of the degenerating oocyte and dying follicular cells.

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Different Follicle-Stimulating Hormone (FSH) sources influence caprine preantral follicle viability *in vitro*

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Introduction

Although the factors that regulate early folliculogenesis are not yet completely established, it is well-known that paracrine and endocrine factors are involved in this phase. Among the endocrine factors, Follicle Stimulating Hormone (FSH) is a heterodimeric glycoprotein synthesized and secreted by the anterior pituitary gland (1). However, the effect of FSH in the *in vitro* follicular culture depends on differences among some factors, such as species, purity degree of commercial preparations of FSH and different *in vitro* culture systems. Furthermore, it was hypothesized that not only the quantity but also the source and the quality of FSH plays an important role in the early follicular phase.

Materials and Methods

Ovaries (n = 8) from 4 adult (1 – 3 years old), mixed-breed goats were obtained at a local slaughterhouse. Caprine ovarian tissues were *in vitro* cultured for one or seven days in Minimum Essential Medium (MEM) alone or containing 10, 50, 100 and 1000 ng/ml of pFSH or rFSH. Control tissues (non-cultured) and those cultured were processed for histological.

Results and discussion

After one day of culture, it was observed a significant reduction ($P < 0.05$) in the percentage of normal follicles in treatments tested compared to control (80%), except when follicles were cultured in 10 and 50 ng/ml of rFSH ($P > 0.05$). In addition, after seven days of culture, the percentage of histologically normal follicles was similar ($P > 0.05$) to control only when 50 ng/mL of rFSH (70.83%) was used. With the progression of the culture from one to seven days, there was a decrease ($P < 0.05$) in follicular viability in MEM^+ alone, pFSH 50 and 1000 ng/ml, as well as in rFSH 10 and 1000 ng/ml. Figure 1 presents normal caprine preantral follicles after seven days of culture with 50 ng/ml of rFSH, showing oocyte and granulosa cells integrity. Moreover, 10 ng/ml of pFSH and all the concentrations of rFSH promoted primordial follicles activation. In conclusion, 50 ng/ml of rFSH maintained the viability of caprine preantral follicles and promoted primordial follicles activation of cultured follicles.

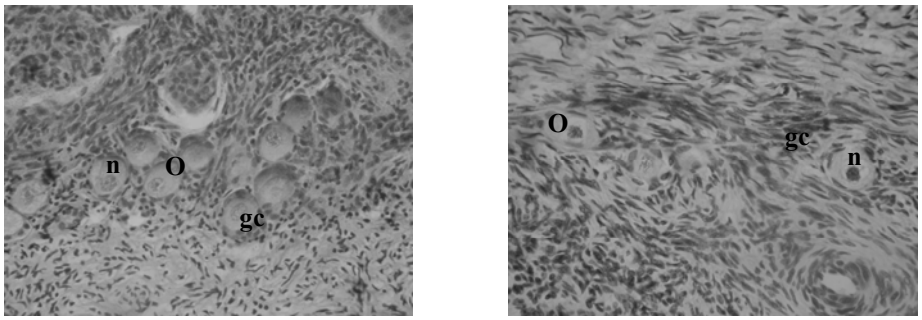

Figure 1. Histological section of (A) culture tissue with rFSH 50 ng/ml (seven days of culture) after staining with periodic acid Schiff-hematoxylin, showing normal follicles and (B) culture tissue with pFSH 100 ng/ml, showing degenerate follicles. **o**: oocyte; **n**: oocyte nucleus; **gc**: granulosa cells (x400).

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The ovarian morphology and follicular maturation of an Amazonian fish *Agoniates halecinus* (Müler & Toschel, 1845)

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Introduction

Agoniates halecinus (Müler & Toschel, 1845), a medium sized freshwater characid known as "sardinha dentuda", can reach up to 20 cm of standard length (1). It is a common species of the Amazon basin, inhabiting mainly river banks and lakes. Despite of no commercial value this species plays an important ecological role as a piscivore (2) and it has a regulating function on the fish assemblage where it occurs. Here we present original results on the ovarian morphology and follicular development of *A. alecinus* from Balbina's reservoir, the largest flooded area due to damming in Central Amazonia.

Materials and Methods

A total of 42 females ranging from 105 to 230 mm in standard length and 53.3 to 293 g in total weight were caught at Balbina's reservoir, in the State of Amazonas, Brazil. The preserved ovaries were embedded in paraffin, sectioned at a 5-7 μ thickness, and stained with Harris Hematoxylin and Eosin (H&E). At least two slides were prepared for each ovary. The description of each oocyte development phase was made according to terminology proposed by teleostenas fishes (3).

Results and Discussion

Along the reproductive cycle, during the different maturation stages, fish ovary changes in size and color. Ovarian histological sections of *A. halecinus* revealed there are no veins and medullar zones as it is expected for most teleosteans (4). The inner wall presents a conjunctive tissue capsule, also called the ovarian tunic, formed by smooth muscle fibers and blood vessels. The first growth phase of the oocyte development is represented by the oogonia, which are the smallest oval cells of the germinative lineages. In the second growth phase, the chromatin nucleolus oocytes are present, and are originated from the oogonia. During the following phases, a large and voluminous nucleus, and several small rounded nucleoli adhering to a small membrane called the cariotheca, can be seen. As maturation advances, a process of vitellogenesis occurs, when the oocytes show extra-vesicular yolk deposition. In the vitellogenic (mature) oocytes, the largest ovarian cells, protein yolk granules (protein vitellus) and cortical vesicles (lipid vitellus) are present. After maturity, follicles break down to allow oocyte release and post-ovulatory follicle formation follows. In the *A. halecinus* ovary, it was possible to observe the presence of atresic follicles, which shows an irregular shape, intense cell disorder with wrinkled follicles, and follicular cell invasions (process of phagocytosis). This degenerative phenomenon can happen in any phase of fish reproductive cycle, however it is more frequent during the late maturity phases or in oocytes not eliminated after spawning. Atresia when occurring in ripe females or mature oocytes is associated to decreasing reproductive potential of a species.

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El Niño influence in the freshwater stingray *Potamotrygon* cf *histrix* reproduction: a histology overview

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Introduction

The freshwater stingrays *Potamotrygon* cf *histrix* is an edemic species of potamotrygonid from Rio Negro Basin, Amazonas State. It is one of the smallest species of Potamotrygonidae family, and it is completely adapted to live in acid and warm water with low oxygen rate. The species exhibit as reproductive mode matrothrophic viviparity with *throphonema*. This species is of particular interest to reproductive studies because its reproduction is strongly affected in El Niño years (1) Histology information of ovary in this species is scarce and these informations can help understand how the reproductive potential of this species will be affected by climate changes in the future years.

Materials and Methods

Fifty females of *P*. cf *histrix* were obtained from Itu River, from 1996 to 2000. The ovaries were dissected out immediately after catching, fixed in Bouin's solution for 24 hours, embedded in paraffin and stained with Hematoxylin-eosin in Manaus-AM. The slides were examined under light microscope.

Results and Discussion

As expected, just the left ovary was functional in this species (2). The ovary surface was covered with the germinal epithelium composed of simple squamous or cuboidal cells. The ovary stroma is formed by connective tissue where the ovarian follicles in different stages including atretic follicles and *corpora lutea* can be observed. The ovarian follicles could be classified into four types: primordial, primary, previtellogenic and vitellogenic follicles. On the other hand, the number of vitellogenic follicles was higher in El Niño years, because higher abundance of food supply at freshwater habitats. Atresy can occur at any stage of follicular development, and it was commom in the reproductive season in the following year right after El Niño. This fact reduced the reproductive potential of this species in those years.

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Ovarian crypts and germinative epithelium in Pinnipeds

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Introduction

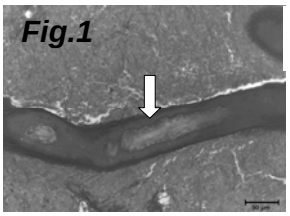
Ovarian crypts were described for many pinnipeds species, besides the bitch others species such as, queen, cow and woman. Their functions are not fully elucidated. Researches suggest that mesenchymal cells in the tunica albuginea are bipotent progenitors for both primitive granulosa and germ cells (1). South–American-fur-seal (*Arctocephalus australis*) reproduction is largely influenced by environmental conditions. The species presents a single annual estrus in early summer, generating only one offspring and shows a rapid corpus luteum involution afterwards. This research intends to elucidate the cellular composition of the ovarian crypts and to study its role in *Arctocephalus australis* ovarian physiology.

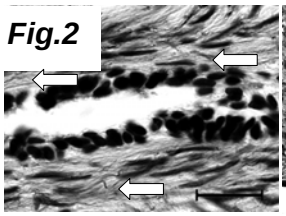
Material and methods

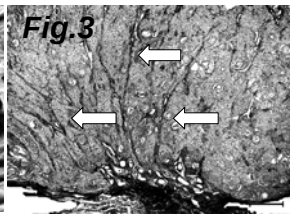
Ovaries of 13 *Arctocephalus australis* (5 adult and 8 young), with death by natural causes were collected in the reproductive colony of Cabo Polônio –Uruguay during 2006 and 2007 (license CITES 000735). The samples were dissected and fixed by infusion of 4% formalin and Karnovsky modified solution. Subsequently, 0.5 cm^3 fragments were obtained for optical and electronic microscopy analysis. Slides were submitted to hematoxylin/eosin, Masson's trichrome, aldehyde/fuchsin and picrosirius staining protocols for microscopical characterization. Immunohistochemistry for Ki-67 nuclear antigen was made in order to determine whether and which ovarian crypt cells are able to proliferate. Two superior canine teeth of each animal were collected for age determination.

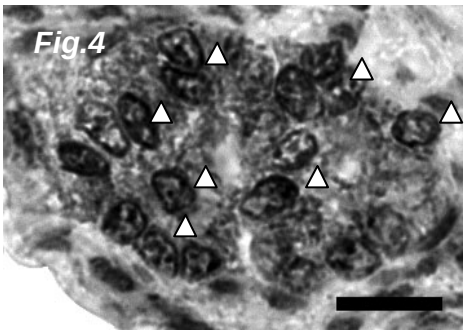
Results and discussion

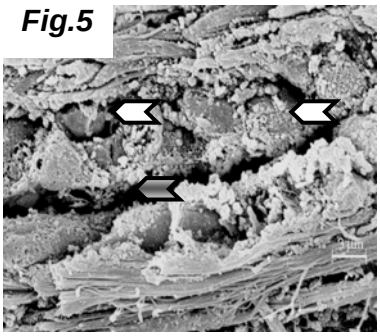
Morphologically ovarian crypts are tubular structure constituted by collagen (Fig.1–Bar 50μm) and elastic fibers (Fig.2–Bar 20μm) distributed in the ovarian parenchyma directed to the tunica albuginea (Fig.3–Bar 50μm). Internally they are covered by germinative epithelium, which express great amount of Ki-67 antigen in epithelial cells (Fig.4–Bar 10μm). Scanning Electronic Microscopic (SEM) showed different sizes of cells and presence of small intra-crypt lumen (Fig.5–Bar 3μm). No important differences were found between the ages studied. Ki-67 staining points towards an involvement of ovarian crypts in ovarian cell proliferation, probably contributing to neo-oogenesis (round shaped cells containing prominent nuclei).











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Ovarian activity induction in puerperium and in post puerperium anestrus Holstein dairy cows by administration of organic phosphorus and vitamin B12 complex

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Introduction

The reproductive efficiency displayed by Brazilian cows is low, with intervals between deliveries nearby 20 months (3). Puerperal and metabolic diseases might be responsible for such lower fertility by lengthening the intervals between delivery and conception (7) as a result of minerals diet deficiency (8). However, animal response in respect to phosphorus (P) administration has been variable, showing to be positive (6) or with no effect (9). The aim of the present study was to induce the return of anestrus cows in lactation towards an ovarian cyclical behavior.

Materials and Methods

A total of 50 Black and White Dutch cows, with a milk production of 11000 liters/305 days were used. The cows were maintained on corn silage, barley residue, "tifton" hay, ration, soybean bran, ground corn, protected fat, sodium bicarbonate, mineral salt and calcareous. Determination of animals in anestrus was based on the following endpoints: the anestrus condition since the delivery and the absence of a dominant ovarian follicle in any of the ovaries established by means of rectal palpation. The treatment protocol was: at the first day, intramuscular (IM) injection of 4 g of organic phosphate added with vitamin B12, 3 g at the third and sixth day after respectively. All animals were treated and separated to one of the three following groups: G1 - 22 animals in anestrus post partum (pp) from 30 to 60 days; G2 - 23 animals in anestrus pp from 61 to 90 days; G3 - 5 animals in anestrus pp from 91 to 150 days. The response of the treatment was considered as the manifestation of visible estrus up to 23 days after injection.

Results and Discussion

The results in regard to the percentual of the animal responses by manifesting the estrus (59.0; 65.2 and 80.0 % in the G1, G2 and G3 groups respectively) did not demonstrate significance between the groups in spite of the statement of Carvalho et al. (2003) in regard to P as being the mineral of the breeding. The animals belonging to group G3 were the ones that produced the best answer to the treatment ($P>0.05$) most probably because were the ones that reached the lactation peak and being in the process of stabilization milk production according account from Frandson et al.(2005). Also, no significance has been found by comparing the groups in regard to the days obtained for the estrus response as a consequence of the treatment employed in the experiment. However, the administration of inorganic P may account for the shortening of the time in which those animals stayed in anestrus (11.4 ± 7.4; 14.8 ± 6.5 and 13.0 ± 9.7 days for the G1, G2 and G3 group, respectively). Better reproductive performance of milk cows was achieved by Lamothe et al. (1976) by the presence of P high levels in the uterine fluids during the estrus. Doyle et al.(1990) found out expressive improvement in the endometrium of cows after administration of P improving the reproduction cyclical characteristic. In conclusion, the IM administration of organic P conjoined with vitamin B12 did not disclose significant effects in regard to the studied reproductive parameters, even though it has been observed a response of the experimental animals to the estrus within two weeks.

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Selection of the future dominant follicle occurs earlier than previously reported for Nellore heifers

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Introduction

In the last five years the quality and definition of ultrasound image has significantly improved, allowing the visualization of smaller follicles than that detected by equipments initially used to characterize follicular dynamics in bovine. In the present work, due to its capacity to measure small follicles (≥1 mm diameter) Aloka 900 ultrasound (transrectal probe 7.5-9.0 MHz, Tokyo, Japan) was used in order to provide additional information about emergence and deviation during the first follicular wave.

Materials and Methods

Nellore heifers (*Bos taurus indicus*, n = 20), were submitted to the following protocol to synchronize ovulation: on Day -10 (D-10) all heifers received a progesterone intravaginal device (1.0 g, DIB®, Syntex, Argentina), previously used for 8 days, and 2.5 mg of estradiol benzoate (EB, i.m., Estrogin®; Farmavet, Sao Paulo, Brazil). The intravaginal device was removed on D-3 and PGF2α was administered (150 µg d-cloprostenol, i.m., Prolise®, ARSA S.R.L., Buenos Aires, Argentina). Twenty four hours afterwards heifers were treated with EB (1.0 mg, i.m.), and from this moment follicular development was monitored by ultrasonography, every 12 hours, until the sixth day after ovulation. The time of follicular deviation was determined by visual or mathematical, Segmented Linear Regression – SLR (1) method. The results obtained by the two methods were compared by Student's t-test.

Results and Discussion

The emergence of the first follicular wave occurred 12 hours before ovulation, and was characterized by the presence of a cohort of follicles (average 10.2 ± 4.08 follicles per heifer). At this time diameter (mean ± SEM) of the largest follicle was 2.7 ± 0.11 as compared to 2.2 ± 0.21 mm of the other follicles. At the time of induced ovulation, i.e., 12 hours after wave emergence, the number of recruited follicles increased to 14.9 ± 2.96. Since there was no difference between visual or SLR method, the data obtained using the visual method will be presented. Follicular deviation occurred 2.3 ± 0.21 days after ovulation, and diameter of the largest (dominant) and second largest follicle were 5.3 ± 0.21 and 4.9 ± 0.05 mm, respectively, at this time. These results about follicular deviation in Nellore heifers are in agreement with data published previously (2). However, in the present work, identification of the future dominant follicle even before ovulation of the previous wave, indicates that selection of the dominant follicle occurs earlier than previously reported for zebu cattle and agrees with recent work published by (3) in *Bos taurus taurus*.

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Fibroblast growth factor 10 (FGF-10) mRNA expression around follicular deviation in *Bos indicus* heifers

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Introduction

Fibroblast growth factors (FGFs) are involved in the regulation of folliculogenesis but their participation in the mechanisms controlling dominant follicle selection has not been assessed. In bovine antral follicles, FGF-10 mRNA was detected in oocytes and theca cells, where expression levels were negatively correlated with estradiol intrafollicular concentrations (1). Furthermore, FGF-10 inhibited estradiol production and expression of AT2 (angiotensin receptor type 2) in cultured granulosa cells (GC; 1,2). In Nellore (*Bos indicus* breed), follicle deviation of the first anovulatory wave occurs on average 2.5 days after ovulation when the dominant follicle presents the mean diameter of 6.0 mm. To gain insight into the involvement of FGF-10 in the mechanisms underlying follicle selection, we assessed thecal FGF-10 expression in the two largest follicles of the wave around deviation in Nellore heifers.

Materials and Methods

The ovarian activity of 10 Nellore heifers was hormonally synchronized and ovulation was detected by ultrasound monitoring every 12 hours. Heifers were then slaughtered 2 (G2; n=4), 2.5 (G2.5; n=3) and 3 (G3; n=3) days after ovulation. The largest and second largest follicles were measured and the theca cell layer was dissected and submitted to total RNA extraction. FGF-10 mRNA levels were measured by real time RT-PCR and normalized by the expression of cyclophilin A (Cyp A). The effect of day on FGF10 expression in the largest and second largest follicles was tested by ANOVA. FGF-10 mRNA levels were compared between the largest and second largest follicles for each day using paired t test.

Results and Discussion

Thecal FGF-10 mRNA expression was lower in the largest follicle compared with the second largest on days 2, 2.5 and 3 ($p<0.05$; Fig. 1). FGF-10 mRNA levels did not significantly vary between days 2 until 3 after ovulation in the largest or second largest follicle ($p>0.05$). In conclusion, present results indicate that thecal FGF-10 expression is decreased in the dominant follicle before deviation suggesting its involvement in the mechanisms controlling dominant follicle selection. As FGF-10 inhibits estradiol production of granulosa cells (1), we propose that FGF-10 is suppressed in the dominant follicle to allow acquisition of full steroidogenic capacity.

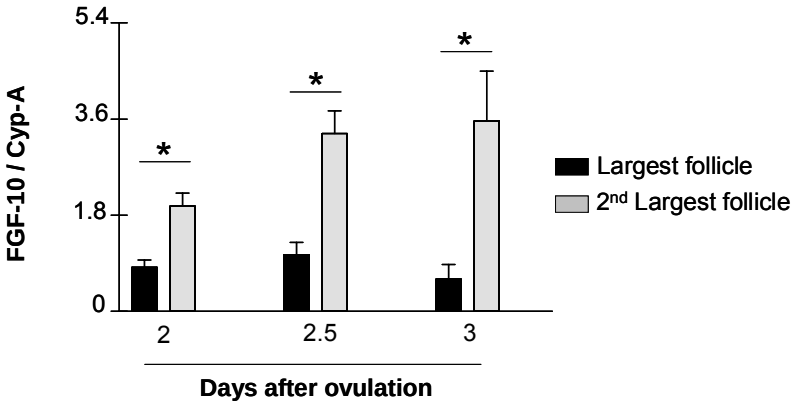

Figure 1. Relative FGF-10 gene expression (mean ± SEM) in dominant and subordinate follicle from bovine CT around follicular deviation in heifers. * Significant differences between follicular status in each day.

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Morphological evaluation of follicle deviation in tabapuã (*Bos indicus*) cows treated with bovine somatotropin (bST)

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Introduction

The selection phase of the dominant follicle (DF) is visualized by ultrasound as an increase in the dominant follicle diameter, while subordinate (SF) ceases its growth or regress. The difference in the growth rate between the two largest follicles of the wave was defined by Ginther *et al.* (1996) as follicular deviation. The bovine somatotropin (bST) is a hypofisarian hormone with multiple effects in growth and cellular differentiation and has shown action in the ovaries especially on follicular growth. Therefore, the objective of this study was evaluated the effect of the bST in follicular deviation in Tabapuã cows.

Material and Methods

There were used 16 Tabapuã cows, with 6 to 7 years of age, and body score from 2.5 to 3.0, maintained in pasture with access to mineral salt Ovarian follicular wave was synchronized with progestagen ear implants and with administration of 1mg of estradiol benzoate IM (day 0) and on day 5, coinciding with the wave emergence, they were divided in 2 groups: G-I (control, n = 8) and G-II (n = 8) treated with 500 mg bST. The bST injections were given subcutaneously in the space between the ischium and tail head. In the day 10 the implants were removed, concomitant the administration of 500 ug prostaglandin. In the day 10, the cows that presented follicles larger than 9 mm, received 300 ug of GnRH for ovulation. The females that ovulated G-I (n = 6) and G-II (n = 5) had their ovaries evaluated by real-time ultrasonography every 12 hours (Honda HS-2000 VET 5 MHz line array transrectaly transducer), during 5 days after ovulation, for evaluation the diameter of the largest dominant and future subordinate.

Results and Discussion

In the G-I one a cow was retreat from the analyses, due to no identifiable deviation. The moment of the visualized deviation was on average (mean ± SEM) at 57.60 ± 4.49 hours on the G-I (control) and at 50.40 ± 4.49 hours in the G-II (bST), or 2.4 and 2.1 days, respectively. In that moment the diamenter of DF and SF were 6.28 ± 0.42 and 6.26 ± 0.41 mm in the G-I and 6.08 ± 4.49 and 6.12 ± 0.39 mm in the G-II. In others studies with zebu females the deviation was observed 2.5 to 2.7 days after ovulation, with the DF measuring of 5.4 to 6.2 mm and the SF 5.3 to 5.9 mm (Sartorelli *et al.,* 2005; Castilho *et al*, 2007; Gimenes *et al.*, 2005). In Holstein females Ginther *et al* (1996) observed the deviation initiating 2.8 days after ovulation with the DF and SF measuring 8.5 and 7.2mm, respectively. The application of bST had not significant effect ($p > 0.05$) in the diameters of the DF and SF, in the moments evaluated. Kozicki *et al.* (2005) working with Holstein cows showed ovulatory diameter significantly larger ($p < 0.05$) in the treated group with bST (18.2 mm) regarding the group control (15 mm). Data of the present study indicate that bST in this dosage not alter the follicular diameter, neither the moment of the follicle deviation in cows.

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Superovulatory treatment increases translation rate of angiogenic factors in buffalo CL

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Introduction

Biotechniques can be an effective way of improving reproduction efficiency and enhancing the production of genetically superior animals. The superovulatory treatment is a common technique aiming to spread desired genetical material, although its use in buffalos still presents limitations, mainly the low embryo recovery rate (1, 2, 3). The corpus luteum (CL) is an endocrine temporary gland that produces progesterone (P) required for the establishment and maintenance of pregnancy and regulation of reproductive cycle. CL development and function are affected by ovarian hyperestimulation (4, 5). The luteal cells of superovulated animals are described to show characteristics compatible with higher protein synthesis (6). The vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are important regulators of CL function and development, and are also affected by superovulatory treatment. Several studies suggest that LH and hCG modulate expression of VEGF and both FSH and LH/hCG receptor-activated pathways are able to induce VEGF mRNA expression in granulosa cells (7, 8, 9). FSH stimulates bFGF receptors (FGFRs) expression and protein synthesis might be involved in the process (10). The aim of this work was to access gene and protein expression of VEGF and bFGF systems in cyclic CL of non-treated and superovulated water buffalos.

Material and Methods

Twelve water buffaloes corpora lutea (six from non-treated animals on day 6 after ovulation and six from superovulated animals in the same day of estrous cycle) were collected at the slaughterhouse, dissected, frozen immediately in liquid nitrogen for posterior protein and RNA extraction or fixed in 4% buffered formalin for posterior immunohistochemistry (IHC). Superovulatory treatment was conducted as described elsewhere (5). Protein expression of VEGF and its receptors KDR and Flt-1 as well as bFGF and its receptors FGFR1-4 was measured by western blotting (WB) and some proteins were also accessed by IHC. For the relative gene expression we used real time RT-PCR. Specifically for bFGF we also carried out an in situ hybridazation in order to localize the mRNA in CL tissue and gain a visual idea about mRNA content in superovulated CL compared to non-treated CL in the same estrous cycle phase.

Results and Discussion

VEGF system and bFGF protein expression accessed by WB and IHC showed an increase ($p < 0.05$) in superovulated CL compared to non-treated CL on day 6 after ovulation (p.o). FGFR-2 and FGFR-3 content measured by WB showed the same increase ($p < 0.05$) observed for the other studied proteins. On the other hand, mRNA expression from all studied genes was decreased (VEGF-A system $p < 0.001$, bFGF, FGFR-1 and FGFR-3, $p < 0.05$) or tended to decrease (FGFR-2 and FGFR-4, $p < 0.1$). The bFGF mRNA expression was detected by in situ hybridization in endothelial as well as luteal cells, and signal was stronger in non-treated animals, which is in agreement with real time RT-PCR data. According to Shikone et al. (10) FSH stimulates FGFRs expression through increase in protein synthesis, once cycloheximide, a protein synthesis inhibitor, could avoid the observed effect. On the other hand, hCG decreases the expression of bFGF transcript in granulosa lutein cells (11) but can also increase VEGF protein expression in CL (12). The interplay between VEGF and bFGF systems may lead to a down regulation of all transcripts once bFGF is decreased. These data indicate that the superovulatory treatment increased translation rate of angiogenic factors in CL.

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Effect of single or repeated insulin administrations before and after mating on litter size in Anglo-Nubian goats

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Introduction

In goats, as well as other ruminants, insulin is a crucial key mediator for reproductive performance, and is involved in the follicular development, steroidogenesis, oocyte maturation and embryo development processes (1). Recent studies reported increasing ovulation rate (1) and viable embryo production in superovulated goats (2) treated with repeated doses of insulin. Moreover, several authors working with cattle suggest that insulin contributes to a better conception rate and to the enhancement of prolificacy (3, 4). However, there is no scientific information available in goats. Thus, the aim of this study was investigate the effect of insulin administration on litter size in Anglo-Nubian goats.

Materials and Methods

Thirty-one Anglo-Nubian goats with similar live weight (32.25 ± 0.77 kg; $P > 0.05$) and age (27.9 ± 0.98 months; $P > 0.05$) (mean ± SEM) had their estrus synchronized by two i.m. administrations of 100 µg cloprostenol, 11 days apart. Twenty-four hours after the second injection, two fertile Anglo-Nubian bucks were mixed with the goats to detect estrus and mating. In this period, animals were treated subcutaneously with human long acting insulin (0.2UI/kgBW/day). Animals from the first group (Ins.I) (n = 10) were submitted to a unique insulin administration at second cloprostenol injection (Day 0). In second group (Ins.III) (n = 11), females received three insulin administrations (Day 0, Day 3 and Day 6), while the goats of third group (Ins.IV) (n = 10) were treated with four insulin doses (Day 0, Day, 3, Day 6 and Day 9). Immediately after kidding, litter size (litter size/dam that gave birth), type of parturition (simple or multiple) and weight at birth were recorded. All animals were maintained in similar feeding and management conditions. The Insulin effect was analyzed by the SAS GLM procedure. Comparison between Insulin means was performed by the Duncan test and between sexes by *t* test. Values were expressed as mean ± SEM.

Results and Discussion

Litter size was similar between groups ($P > 0.05$) showing a mean respectively for Ins.I, Ins.III and Ins.IV of 2.0 ± 0.45, 1.6 ± 0.24 and 1.6 ± 0.24. These data are not in agreement to (3) that working with cattle, verified that continuous elevations in plasma insulin rates are more important than the progesterone to the early embryo development and, consequently, to obtain greater prolificacy rates. Other authors (4) reported in lactating dairy cows that IGF-I, positively correlated to the plasma insulin rates, increases embryo survival and, thus, the prolificacy rates. Our findings also show a similar proportion of multiple parturition type ($P > 0.05$) (mean 58.82 ± 12.30%) and for the weight at birth between sex in each group ($P > 0.05$) and among groups in female kids (mean: 2.88 ± 0.06 kg; $P > 0.05$). However, in the male kids Ins.I had a lower weight at birth when compared to Ins.IV (2.77 ± 0.53 kg vs. 3.23 ± 0.16 kg; $P < 0.05$). Based on our results, it was concluded that single or repeated insulin administrations before and after mating did not affect the litter size in Anglo-Nubian goats.

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Postpartum ovarian activity by endocrine profiles in Colombian Sanmartinero cows

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Introduction

There are at least seven Colombian native breeds of cattle spread along the country. Several studies have been made regarding some reproductive aspects in Costeño Con Cuernos (1) and Romosinuano (2, 3) breeds. Those animals have shown adaptation to the environmental conditions in tropics, in terms of reproductive performance. Previously works about luteal function and follicular dynamics associated with the first spontaneous estrous cycles in postpartum cows have been made (4). The aim of this study was to describe levels of progesterone and estradiol associated with ovarian function during postpartum period in Sanmartinero cows.

Materials and Methods

15 suckling cows calving simultaneously were studied since day 7 postpartum, and during all over their postpartum period. Blood samples were taken three times a week and progesterone (P4) and estradiol (E2) serum levels during the onset of estrous activity were measured by radioimmunoassay, using human commercial kits (MP Biomedicals Inc, Costa Mesa, CA). No extraction was made. Body condition was evaluated, in a five-point scale [1 = emaciated to 5 = obese, (5)], in order to determine the energy balance.

Results and Discussion

Minimum energy balance (nadir) was presented approximately on day 60 after calving (Fig. 1), being coincident with the interval from parturition to first postpartum ovulation (60.6 ± 24.4 days). All cows had a short luteal phase after first ovulation, with an average interval of 7.18 ± 0.75 days between first and second ovulation that was associated with less progesterone than was second ovulation (Fig. 2). These results are in agreement with those found in other Colombian native cattle (1). Signs of estrus were less obvious or absent preceding first ovulation. Estradiol profiles evidenced the presence of follicular waves since early lactation as estradiol waves due to secretion by follicles. Estradiol levels tended to increase through lactation, from 26.43 ± 5.6 pg/ml between first and second weeks after calving, to 43.76 ± 6.5 pg/ml at the first ovulation (R^2 = 0.436) in a consistent pattern (Fig. 2). This study allows confirming previous findings regarding some aspects of endocrine patterns during postpartum period and its relation with nutritional status.

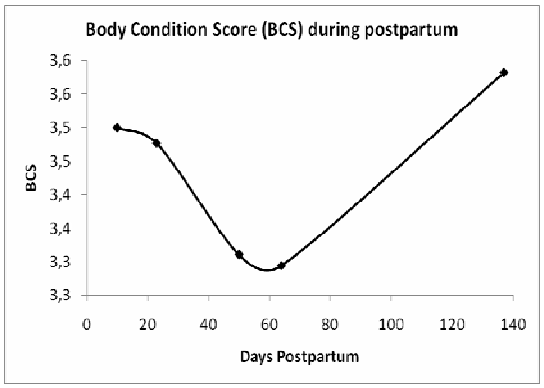

Figure 1. Average body condition score

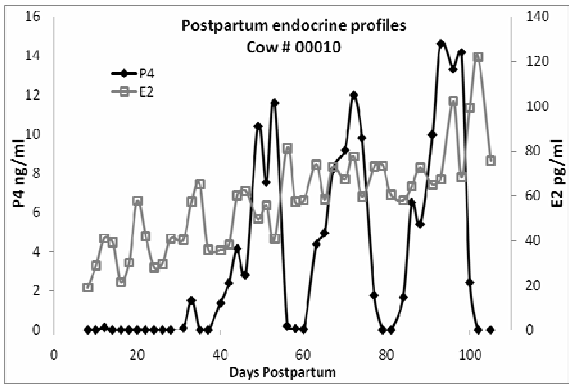

Figure 2. Postpartum endocrine profiles

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Rate and time to ovulation in Nelore cows treated with estradiol Cypionate or Benzoate to induce ovulation on FTAI protocols

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Introduction

Hormonal treatments were developed to control as the luteal as follicular function providing the synchronization of ovulation, allowing the fixed-time AI (FTAI) without estrus detection (1). In conventional FTAI protocols that use estradiol benzoate as ovulation inductor (24 hours after device withdrawal) four managements are necessary for hormones injection and artificial insemination. The aim of this study was to evaluate the follicular dynamics in Nelore cows (*Bos indicus*) treated with estradiol Cypionate (EC) or Benzoate (EB) as ovulation inductor in order to minimize the number of handling during these protocols.

Material and Methods

At this Experiment, it was used 64 Nelore cows with body condition score (BCS) of 2.70 ± 0.25 (1 to 5 scale). At day 0 (AM), all animals received 2 mg of EB (Estrogin®, Farmavet, Brazil) and a Norgestomet ear implant (Crestar®, Intervet, Netherlands). On Day 8, the cows were allocated in one of four groups, considering the BCS and cyclicity status. At this day, the implant was removed, 150 µg of Cloprostenol (Preloban®, Intervet, Netherlands) and 300UI of eCG (Folligon®, Intervet, Netherlands) were administered in all animals (AM for EC8, EB9 and EB8 groups and PM for EB8.5 group). The cows of group EC8 (n = 15) received 1.0mg of EC (ECP®, Pfizer Saúde Animal, Brazil), the cows of EB8 (n = 15) and EB8.5 (n=15) groups received 1.0mg of EB on device withdrawal [AM for EC8 and EB8 groups or ten hours later (PM) for EB8.5 Group]. The cows of Group EB9 (n = 12) were treated with 1.0mg of EB 24 hours after the device removal (D 9). Ultrasound (Chison 600VET) exams to monitor follicular dynamics occurred every 12h from device withdrawal until ovulation. The statistical analysis was accomplished by GLM procedure of the Statistical Analyses System.

Results and Discussion

The results for EC8, EB9, EB8 and EB8.5groups are presented in Table 1. These results are in agreement with (2) data that found no difference in ovulatory follicle diameter and ovulation rate between estradiol benzoate at implant withdrawal or 24 hours after the removal. They also found difference in time to ovulation as we in this study. Additionally, (3) did not find difference in these variables when estradiol benzoate 24 hours after removal was compared to estradiol cypionate at device withdrawal.

Table 1. Diameter of the ovulatory follicle, ovulation rate and interval from device removal to ovulation for EC8, EB9, EB8 EB8.5 groups.

	EC8	EB9	EB8	EB8.5	P
Diameter of the ovulatory follicle (mm)	13.9 ± 0.8	12.8 ± 0.7	12.4 ± 0.6	12.9 ± 0.5	0.42
Ovulation rate (%)	73.3 (11/15)	93.3 (14/15)	93.3 (14/15)	89.5 (17/19)	0.78
Interval from device removal to ovulation (h)	75.3 ± 3.3[a]	73.8 ± 1.8[a]	56.3±2.5[b]	56.5 ± 1.9[b]	<0.01

Conclusion

These results show that EB on EB8.5 protocol and EC could be used for inducing ovulation, allowing a reduction in the number of animal handling and the FTAI during all day. Moreover, when the implant withdrawal was performed with a delay of 10 hours plus estradiol benzoate injection (EB8.5 protocol), the ovulation period was similar to EC8 and EB9 groups.

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Effect of type of semen (sexed vs non-sexed) and time of AI (60h vs 64h) on pregnancy rates of postpartum Nelore cows inseminated in a fixed time

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Introduction

Although the use of sexed-sorted sperm in AI cattle industry is becoming more common in recent years, fertility results appear to be lower when compared with regular non-sexed sperm (1). Many factors might be causing lower pregnancy outcomes in cows inseminated with sexed sperm including semen processing (1), animal category (2), bull effect and sperm dose (2), and interval between insemination and ovulation (3) probably due to the reduced life span in the uterine tract of the sex-sorted spermatozoa (4). Previous results from our research group (4) indicated that a 6h delay in the timed AI (from 54h to 60h after P4 implant removal) seemed to improve pregnancy results of beef cows inseminated with sexed sperm. Thus, the objective of this study was to evaluate the effects of delaying 4 more hours in the interval between norgestomet ear implant removal and FTAI on pregnancy rates in *Bos indicus* (Nelore) beef cows inseminated with sexed or regular (non-sexed) sperm.

Material and Methods

This experiment was performed during December of 2007, in two commercial farms in Parana State/Brazil. On Day 0, Nelore cows (n=383) from 35 to 65 days postpartum, received 2 mg of estradiol benzoate (Estrogin®, Farmavet, Brazil) and norgestomet ear implant (Crestar®, Intervet, Brazil). On Day 8, the implant was removed and 400UI of eCG (Folligon®, Intervet, Brazil) plus 0.150mg of D-Cloprostenol ($PGF_{2\alpha}$, Preloban®, Intervet, Brazil) were administered. On day 10 (48h after implant removal), all animals received GnRH (100µg, Fertagyl®, Intervet, Brazil). Then, cows were homogenously divided in 4 experimental groups: 1) C60h – insemination with regular sperm ($20x10^6$ sperm/dose; n = 91) 60h after Crestar withdrawal; 2) S60h – insemination with *X*-sorted sperm ($2.1x10^6$ sperm/dose; n = 100) 60h after Crestar withdrawal; 3) C64h – insemination with regular sperm ($20x10^6$ sperm/dose; n = 99) 64h after Crestar withdrawal; 4) S64h – insemination with *X*-sorted sperm ($2.1x10^6$ sperm/dose; n = 93) 64h after Crestar withdrawal. One Nelore bull was used to inseminate all cows, and each ejaculate was proportionally divided to make the same amount of doses of sexed and regular sperm. Pregnancy exams were performed 30 days after AI with ultrasound.

Results and Discussion

There was no interaction between type of sperm and time of AI ($P > 0.10$). The analysis of the main effects showed that the type of sperm affected pregnancy results (Regular: 55.3%^{a} vs Sexed: 40.9%^{b}, $P < 0.05$). In contrast, delaying the time of AI did not significantly affected pregnancy results (60h: 50.8% vs. 64h: 45.3%, $P > 0.10$). Thus, pregnancy rates after using sexed sperm in postpartum Zebu cattle inseminated in a fixed time produced acceptable results. However, pregnancy rates with sexed sperm could not be improved with the further increase of 4h in the interval between the Norgestomet ear implant removal and the FTAI.

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Estrous and ovulation rate after synchronization of nulliparous Anglo-nubian goats treated with increasing insulin doses

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Introduction

The lower reproductive response of nulliparous at first mating is a recognized limiting factor of goat husbandry. Several studies showed in adult goats that insulin administration can have a positive action on ovarian function, increasing numbers of recruited follicles and ovulation rate (1, 2). However, these studies did not appointed any evidence about the reproductive response to insulin administration in young animals. Thus, the aim of this work was to compare the response of estrous synchronization and ovulation rate in nulliparous Anglonubian goats treated with increasing insulin doses.

Materials and Methods

The experiment was conducted in Tauá-CE (6, 0°S and 40, 4°W). Fifteen Anglo-nubian nulliparous goats with live weight of 26.60 ± 4.70 kg (mean ± SEM) and aged between 8 and 15 months has their estrous synchronized with 60 mg MPA vaginal sponge for 10 days and 50 μg of cloprostenol on 8th day. Goats were allocated in three groups: Insulin I (n = 5), Insulin II (n = 5) and Control (n = 5). In Insulin I and II groups, all animals were treated subcutaneously with 0.14 IU/kg BW/day and 0.2 IU/kg BW/day human long acting insulin, respectively, during three consecutive days beginning 48h before sponge removal. In control group saline solution was administrated. The goats were monitored three times a day (06:00, 12:00 and 18:00 hours) for the occurrence of estrous and mated at onset of estrous and again 24 h later, using three Anglonubian bucks of confirmed fertility. Ovulation rate was recorded 3 days after mating, using a Falco 100 ultrasound scanner (Pie Medical®, Maastricht, Netherlands) fitted to 6/8 MHz linear-array probe. The insulin effect was analyzed by the SAS GLM procedure. Comparison between groups was performed by the Duncan test. Values were expressed as mean ± SEM.

Results and Discussion

All goats showed estrus after synchronization treatment. Estrus length, the interval between sponge removal and estrus onset as well as ovulation did not differ between groups ($P < 0.05$), with a pooled mean, respectively of 37.80 ± 1.93; 28.67 ± 2.12; 59.80 ± 2.18 hours. These data are in agreement with (1). Higher induction of estrous has been reported in insulin-treated anestrous cattle (3) and goat (2), suggesting an insulin effect on steroidogenesis. Ovulation rate was significantly ($P < 0.05$) higher in the Insulin I than in the Control group (2.6 ± 0.51 vs. 1.0 ± 0.2; $P < 0.01$), while in Insulin II group was similar to other treatments (1.6 ± 0.4). Similar findings in goats treated with insulin prior superovulation have been reported (1). The positive effect of insulin on ovarian response in low dose insulin group should be due to either an increase of gonadotrophin-dependent follicles number (4) as well as reduction of follicular atresia (5). Based on our results, we can conclude that treatment with increasing insulin doses in nulliparous goats did not affect the response to estrous synchronization; however the administration of 0.14 IU/kg BW/day produced a higher ovulation rate.

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Preovulatory follicle dynamics in Toggenburg goats after treatment with intravaginal implants of progesterone for 6, 9 or 12 days

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Introduction

The use of ultrasound in reproduction made the daily monitoring of ovarian follicular development during estrous cycle possible, demonstrating the growth and regression patterns of antral follicles, and allowed a clear understanding of the process of recruitment, selection and follicular dominance in cattle (1,3) and sheep (2). The aim of this study was to evaluate the preovulatory follicular dynamics in goats, after hormone treatment at the anoestrus period for the induction and synchronization of estrus.

Material and Methods

Nulliparous and pluriparous (lactating or not) Toggenburg goats, with mean weight of 47.37 ± 1.75 and 3.65 ± 0.15 of body score condition (1 to 5 range), were selected and equally distributed into three groups: Treatment 1 (n = 10); treatment 2 (n = 10) and treatment 3 (n = 10). The animals remained with the intravaginal progesterone device (CIDR ®, Pfizer - New Zealand) for 6, 9 or 12 days, respectively. At the moment of insertion of the device they received 1ml of prostaglandin (Lutalyse®, Pharmacia Corporation-USA) paravulvar and 24 hours before the device withdrawal 200U.I. (IM) equine chorionic gonadotropin (Novormon®, Syntex – Argentina) was given. The preovulatory dynamics was monitored using a portable ultrasound device equipped with an adapted 5 MHz probe, at 8-hour intervals (08:00 a.m., 04:00 p.m. and 00:00 a.m.) from the moment of CIDR withdrawal until the confirmation of ovulation. The position and number of ovarian follicles were recorded, and the diameter of antral follicles larger than 3.0 mm measured. Data from each animal was recorded on individual cards.

Results and Discussion

There was a higher incidence of ovulations in the right ovary in all treatments. The 9 day treatment presented larger ($p < 0.05$) follicles at the moment of the CIDR withdrawal. As the follicle growth rate, follicle size at ovulation and number of ovulations were similar among treatments ($p > 0.05$), the difference in dominant follicle size at CIDR withdrawal resulted in a shorter interval to ovulation in the 9 day treatment. This difference may be related to the moment of the follicle emergence when the insertion of the device, and also the follicle turnover period in goats (Table 1).

Table 1. Preovulatory follicle dynamic characteristics in each treatment.

Parameter	Treatment		
	12 days	9 days	6 days
Follicular diameter at the time CIDR withdrawal (mm)	5.6 ± 0.9[a]	6.7 ± 0.88[b]	5.6 ± 0.7[a]
Follicular diameter at the time ovulation (mm)	7.1 ± 0.6[a]	7.2 ± 0.56[a]	6.7 ± 0.6[a]
Interval CIDR withdrawal – ovulation (h)	37.1 ± 9.3[a]	21.9 ± 5.8[b]	38.5 ± 8.3[a]
Growth follicular rate (mm/day)	1.0 ± 0.5[a]	0.5 ± 0.9[a]	0.7 ± 0.5[a]
% of ovulation in right ovary	64.0[a]	62.0[a]	69.0[a]
Total of ovulations	1.8 ± 0.9[a]	1.3 ± 0.5[a]	1.6 ± 0.5[a]

[a,b]Means followed by different letters, in the same line, differ ($p < 0.05$).

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Influence of the temperature on the final oocyte maturation induced by hypophysation in *P. argenteus*

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Introduction

Final oocyte maturation (FOM) and ovulation are critical points for fish cultivation. Temperature acts at different levels of the reproductive process, interfering in the sequence of events and time of FOM and ovulation (1). Variations in temperature can cause changes in the dynamics of cytoskeleton elements, leading to microtubules aggregation, when the temperature is higher and its breakdown when temperature is lower, interfering in the nuclear migration during FOM (2). The aim of this experiment was to analyze the sexual steroids levels and the cytoskeleton organization on the FOM; spawning and fertilization of *Prochilodus argenteus* submitted to spawning induced by hypophysation in two temperature regimes.

Material and Methods

The experiments were conducted in the Hydrobiology and Hatchery Station of Três Marias, MG –CODEVASF, in two reproductive seasons. In each experiment were used 30 females in advanced maturation, which were captured in São Francisco River and acclimated during 2 months in cultivation tank. The fish were transferred to tanks with continuous flow of water, and separated into two groups of 15 individuals (group A = 22-23 ºC; group B = 25-26 ºC). Both groups were submitted to spawning induced by hypophysation using two doses of crud carp pituitary extract (CCPE). To examine the serum profiles of sexual steroids, blood samples were collected in the following times: 0h, 14h, 23h, 25h and 26h after the first CCPE dose. Fecundity was determined considering the weight of spawned oocytes and the number of oocytes per gram of spawned oocytes. The males received a single dose of CCPE. After fertilization, the eggs were placed in incubators for development. The fertilization rates were determined after the blastopore closure in at least 100 eggs. The larva abnormality rates were obtained in at least 50 recently hatched larvae of each sample.

Results and Discussion

The final oocyte maturation process occurred similarly in both groups, although in Group A (22-23 ^{o}C) this process was slower, with a delay of approximately 2 hours. Moreover, several oocytes of the group A didn't complete the FOM. Germinal vesicle migration toward the micropyle started after the first CCPE dose and germinal vesicle breakdown occurred after the second CCPE, similar to findings of other authors (3). At this stage, the chromosomes in metaphase were near the micropyle. Spawning occurred in 100% of females of the group B and 67% of the group A. The fertilization rates were above 80% and the larval abnormalities were below 10%, with no statistical differences between the groups. Fecundity was higher in the group B (25-26 ^{o}C). The profiles of sex hormones showed similar tendency in the two groups and with others studies (3). An increase in the testosterone levels occurred after the first dose of CCPE, decreasing before spawning. The 17β-estradiol levels exhibited few variations during FOM, and the 17α-hydroxyprogesterone (17α-P) levels increased at the time of spawning and declined afterward. Group A presented a late elevation peak of 17α-P, and thus the spawning was delayed. Immunohistochemistry reactions showed tubulin associated to the germinal vesicle and an actin ring in the cortex probably responsible by the ooplasmic segregation and fertilization events. In conclusion, the results indicated that lower temperatures impair the final oocyte maturation and spawning of *P. argenteus* under cultivation.

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Effects of estradiol-17β on ovarian function and pregnancy in Nelore cows

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Introduction

Estradiol is capable to induce follicular atresia and recruit a new wave of follicular growth within a known interval (1). Therefore, strategic administration of exogenous estradiol-$_{17\beta}$ could prevent the presence or action of a dominant follicle (DF) during the critical period for maternal recognition of pregnancy (CP). Estradiol produced by DF plays a key role in triggering luteolysis (2) and preventing gestation in the cow. This study aimed to assess the effects of the exogenous administration of estradiol-$_{17\beta}$ on ovarian function and its implications on the maintenance of pregnancy in Nelore cows.

Material and Methods

130 Nelore cows (BW=412.7 ± 33.9 kg and BCS = 5.5 ± 0,5) were treated with the OvSync protocol, which consisted in the administration (IM) of 8μg of Buserelin acetate (GnRH analogous) followed by a 0.150 mg of d-Cloprostenol ($PGF_{2\alpha}$ analogous) seven days later and another Buserelin shot 48h after. Then, cows received nothing else (G_C; n = 65) or 5mg of estradiol-$_{17\beta}$ (E_2) on D_{12} after estrus (GE2; n = 65). In the 1st trial 10 cows from each group were submitted daily to ovarian ultrasonography and determination of plasma progesterone concentration ($[P_4]$) since the 2nd shot of GnRH until the subsequent natural ovulation. Progesterone concentrations were obtained through a validated RIA and samples were run in the Laboratório de Dosagens Hormonias – LDH of the FMVZ of Universidade de São Paulo. In the 2nd trial, the remaining cows (55 each group) were artificially inseminated by appointment (TAI) at 16h after the 2nd GnRH administration. Pregnancy rates (PR) were determined by ultrasound.

Results and Discussion

Estradiol efficiently synchronized the emergency of a new wave (4.0 ± .69 days) and reduced ($P < .05$) the time within the CP under the influence of a DF (3.5 ± .8 and. 4.7 ± .8 days respectively to G_{E2} and G_C from D_{15} to D_{20}). Interovulatory interval, mean number of follicular waves of development, maximum size reached by corpus luteum, interval from luteolysis to estrus and diameter of preovulatory follicle were not affected ($P > .05$) by treatment. Cows receiving estradiol$_{17\beta}$ showed maximum $[P_4]$ 12.0 ± 0.6 days after estrus ($P < 0, 01$) compared to 13.4 ± 0.6 for control cows. Cumulative value for $[P_4]$ throughout the luteal phase was also lower ($P < 0, 01$) to G_{E2} (24.2 ± 6.7 ng/mL) compared with 34.8 ± 6.7 ng/mL to Gc. Luteolysis (when $[P_4]$ <1.0ng/mL) took place earlier in cows treated with estradiol (17.0 ± .57 days) if compared to control (18.2 ± .57 days) cows. Similarly, estrous cycle length was shortened (19.3 ± 1.78 days) in G_{E2} cows as compared to G_C (23.4 ± 1.38 days). In addition, DF size at luteolysis was too small to ensure the ovulation of a fully developed oocyte for G_{E2} cows (7.8 ± 1.75 mm) when compared to the normal value observed for G_C (9.6 ± 1.75 mm). Pregnancy rates (PR) at TAI were: 34.5% and 5.4% respectively for G_C and G_{E2}. PR at TAI for G_{E2} was lower ($P < .05$) than G_C. In summary, 5mg of estradiol-$_{17\beta}$ given 12 days after TAI caused pregnancy termination in the cows and significantly reduced PR. The same protocol given to non-inseminated cows promoted not only atresia of the DF but also induced luteolysis and in the cows. However, three cows (5.4%) were refractory to damaging effects of estradiol, once they maintained their gestation. It is fair to assume that luteolysis did not occur in these cows. It remains to be determined why these cows showed anti-luteolytic response strong enough to ensure the gestation to proceed. It is speculated the role of the conceptus itself in preventing the deleterious action of estradiol-$_{17\beta}$. In conclusion despite of the fact that estradiol-17_{β} given on day 12 of estrous cycle was capable to reschedule ovarian function, it also promoted luteolysis, caused embryonic losses and therefore it is not recommended.

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Progesterone profile secretion by *Corpus luteum* formed after follicular aspiration in mares

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Introduction

Follicular aspiration has been used to study follicular dynamics and ovulation synchronization in animals. This study aimed to evaluate the development of the *Corpus luteum* (CL) following follicular aspiration in mares. Luteal development was determined by measuring serum concentrations of progesterone (P_4), and by a subjective characterization of the CL.

Materials and Methods

Crossbreed mares (n = 26) from 5 to 22 years of age, weighing 350 to 500kg and kept on pasture, were used for follicular aspiration. Ovarian activity was accessed by the use of transrectal examination. Mares with follicles ≥25 mm (regardless the oestrus cycle status) were assigned to groups according to follicular diameter: 25-29 mm; 30-35 mm and >35 mm. Transvaginal follicular aspiration (1) was performed in all follicles ≥10 mm. Echographic images of each follicle and structures at 4 days after aspiration were subjectively evaluated, considering the echographic image (Fig. 1). Blood samples were collected daily starting immediately before (D0) and until 8 days after aspiration (D8), and stored at -20°C for progesterone radioimmuneassay (RIA) evaluation. Linear regression analysis was used to establish the curve of progesterone production. Qui-square test was used to verify the luteinization rates while Kruskal-Wallis to analyze whether the echographic score was a valid method to indicate the functionality of the CL.

Results and Discussion

The average diameter and the number of aspirated follicles were 26.3 mm ± 1.38 (n = 7); 32.7 mm ± 1.28 (n = 8) and 43.2 ± 5.40 mm (n = 11) for the groups with diameters 25-29 mm; 30-35 mm and >35mm, respectively. During the aspiration, the fluid flow was observed until the moment the follicle collapsed. No secondary ovulations were detected on transrectal ultrasonography during the first 8 days after ovulation. The evaluation of luteal activity was based on echographic patterns in the presence of a desirable P_4 production. The luteinization rate based on echographic characterization was highly significant ($P < 0.0001$), proving that the US evaluation was valid method for CL classification. Corpora lutea classified as CL 1 and CL 2 had declining concentrations of P_4 at a rate of 0.25 ng/mL/day ($P = 0.03$) and 0.14ng/mL/day ($P = 0.2$), respectively. In the mares with CL 3, the P_4 concentration increased (0.61 ng/mL/day; $P < 0.0001$). This pattern is not different from those reported in mares with spontaneous ovulation. It was concluded that the echographic score was a practical and efficient method to confirm luteinization. Mares that had an echographic score of P_4 "CL 3" after aspiration might be suitable as recipient mares for embryo transfer.

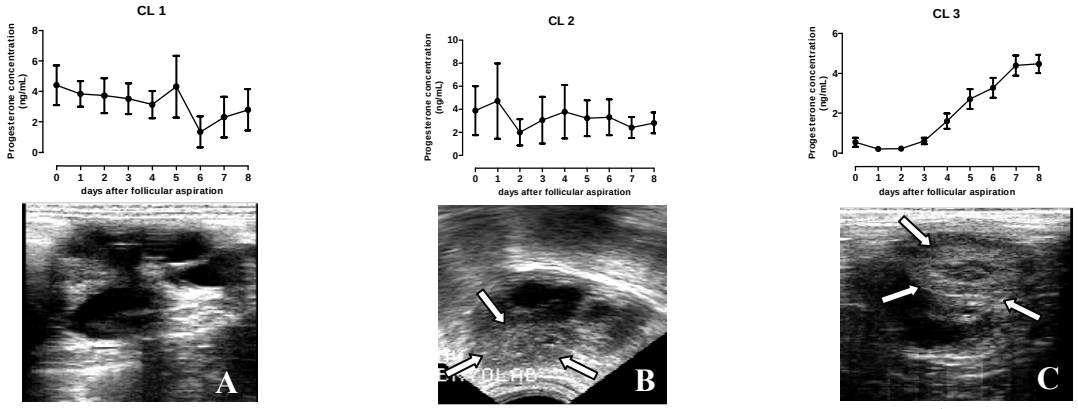

Figure 1. *Corpus luteum* (CL) classification in crossbreed mares according to ultrasonographic pattern at the 4th day afterr follicular aspiration (D0 = Follicular aspiration day). (A) CL 1– No CL visualization until the 4th day after aspiration; (B) CL 2– Luteal structure formed, however not much evident and with low echogenicity (see blue arrows). (C) CL 3– Image compatible with the CL (luteal structure with high echogenicity, see white arrows).

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Ultrastructural effects of phosphodiesterase-5 inhibitor in luteal cells

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Introduction

Adenosine 3', 5'-cyclic monophosphate (cAMP) and guanosine 3', 5'-cyclic monophosphate (cGMP) are ubiquitous nucleotides that, in concert with calcium and IP3, orchestrate intracellular signaling. Cyclic nucleotides cGMP and cAMP have different effects depending of the cell type. In the ovary, the role of cAMP is well known, and the actions of LH and FSH on ovarian functions are believed to be mediated in large part through its increased production. On the other hand, cGMP concentrations are regulated in an inverse manner compared to cAMP in the ovary, in certain phases of cycle (1). Many studies have reported effects of cGMP on ovarian cells, suggesting that cGMP may be a necessary component as both a growth enhancer and a survival factor (2, 3). In human, bovine, and rat smooth muscle, PDE5 was purified and characterized as a cytosolic PDE that specifically hydrolyses cGMP. Use of phosphodiesterase-5 inhibitor (iPDE5) increases intracellular cGMP levels. In the present study, we attempted to evaluate use of iPDE5 on ovary cells.

Materials and Methods

Twenty-four adult female *Swiss webster* mice, 45-day-old, were used in all experiments. One experimental group composed by twelve animals received 5 mg/kg body weight of Vardenafil for 30 days per os in aqueous solution in water bottle. The control group also composed by twelve animals received only pure water. After treatment the experimental and control animals were killed and pieces of ovary were quickly excised with a scalpel and fixed for electron microscopy evaluation.

Results and Discussion

Luteal cells from control group showed lipid droplets and mitochondria with tubular cristae. Ribosomes were visible near to the nucleus. Luteal cells from vardenafil-treated group showed numerous mitochondria, and paucity of lipid droplets, comparing to control group. Several ribosomes were present in cytoplasm, alone and attached to the endoplasmic reticulum. Size of luteal cells seemed to be different too. To certify, we measured the medium diameter of 40 cells (20 for each group), and compared groups. Mean diameter of control cells (13.088 ± 1.538 µm) was considerably larger than vardenafil-treated group (10.638 ± 1.386 µm) ($t_{(0.05;\ 38)} = 5.2921$; $p < 0.0001$). Also, control cells had statistically significant higher number of mitochondria (Control: 105.1 ± 37.087. Vardenafil-treated: 63.35 ± 23.979) ($t_{(0.05;38)} = 4.228$; $p < 0.0001$) and lipid droplet (Control: 54.55 ± 27.53. Vardenafil-treated: 24.15 ± 12.38) ($Z(U)_{(0.05;38)} = 3.828$; $p < 0.001$). To compare data of cell diameter and number of mitochondria we used t test; however, to compare number of lipid droplet we used MannWhitney test, because variance was heterocedastic. Morphological changes suggest that use of iPDE5 probably affects hormonal pathways.

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Occurrence of subnormal corpus luteum in superovulated Santa Inês sheep using protocols with or without LH administrated at the end of the FSH treatment

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Introduction

Corpora lutea (CL) that are small (<5mm), grossly pale, with little or no protrusion from the surface of the ovary are defined as subnormal and are associated to low circulating progesterone concentrations. The formation of a subnormal or short-lived CL has been widely reported in superovulated sheep (1). Two causes of subnormal CL formation have been proposed: i) inadequate follicle development preceding luteal gland formation (2), and ii) premature activation of the luteolytic mechanism (3). Based on the latter possibility in practice, luteolytic inhibitors such as Flunixine Meglumine are used. Thus, the objective of the present study was to evaluate the occurrence of subnormal CL in superovulated Santa Inês sheep using protocols with or without LH administrated at the end of the FSH treatment.

Materials and Methods

Twenty multiple ovulations were accomplished and the experiment was designed as a 2 by 2 factorial, with the main effects of replicate and treatment. The estrus was synchronized with a progesterone-releasing intravaginal device (CIDR™; Pfizer-New Zealand) inserted on Day 0, replaced by a new one that was maintained from Day 7 to Day 14. Two doses of the 37.5 µg of D-cloprostenol (Prolise™, Arsa - Argentina) were administered, IM, on Day 7 and 14. Then, 256 mg of FSHp (Folltropin™, Bioniche-Canada) were administrated in 8 decreasing doses, starting on Day 12. On Day 14, all females received 200 IU of eCG (Novormon™, Syntex-Argentina). On Day 15, the animals were homogeneously allocated in one of the two experimental groups: Control (GC) and treated (G-LH). Sheep in GC did not receive exogenous LH, while sheep in G-LH were treated with 7.5 mg of LH (Lutropin™, Bioniche-Canada), 24 h after device withdrawal (Day 15). On Day 16, the number of ovulatory follicles was verified by laparoscopy. On Day 17, 18 and 19, all females received 75 mg of Flunixine meglumine (Banamine™, Shering-Plough, Brazil). On Day 21, the ovarian structures were evaluated by laparoscopy. Number of normal CL, subnormal CL and anovulatory follicles were measured. Two-way analysis was run for the effects of replicate (2 replicates) and groups (GC and G-LH). Data were analyzed using by ANOVA using procedure GLM of SAS, and means (±SD) were compared using Kruskal-Wallis test ($P < 0.05$).

Results and Discussion

Ovulation rate tended to be increased in G-LH (85.44% *vs* 77.77%, $P = 0.08$). The number of CL (mean ± SD) was 10.5 ± 3.8 in GC and 13.5 ± 4.84 in G-LH; $P > 0.1$. There was not effect of replicate evaluating the number of CL in GC (12 ± 3.46 vs 9 ± 3.87); whereas in G-LH there was a negative effect (16.6 ± 2.97 vs 10.4 ± 4.45). The number of anovulatory follicles did not differ statistically between groups (GC: 3.0 ± 3.19; G-LH: 2.3 ± 1.63). Likewise, no effect of repeatability was shown for this variable. The anovulatory rate tended to decrease in G-LH (22.22% *vs* 14.55%, $P = 0.08$). Even using Flunixine Meglunine to prevent luteolysis, 20% of the ewes treated with GC and 40% of the ewes in G-LH presented at least one subnormal CL ($P > 0.05$). The numbers of subnormal CL were 3.0 ± 1.41 for GC and 1.25 ± 0.5 for G-LH ($P > 0.1$). Failure in formation of CLs was not punctual characteristic because it was found that a single female had normal and subnormal CL. These data does not corroborate with data published by Rubianes et al. (1996). The subnormal CLs were observed just in second replicate of each female. This fact suggests that the repeatability of treatment could have interfered in formation of CL, however, statistical difference was observed just for number of subnormal CL in G-LH. The greater number of subnormal CL in G-LH in the second reply might have been caused by the administration of LH, which can induce the ovulation of a follicle without appropriate capacity of formation of a CL. Indeed, a previous study showed that GnRH administration when follicles are not completely maturated induce the formation of CL without adequate function or short lived (4). The results showed that the formation of a subnormal or a short-lived CL can be more related to the incapacity of the ovulated follicle to form a normal CL than to the premature activation of luteolytic mechanism.

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Expression of fibroblast growth factor homologous factors (FHFs) during bovine luteal development

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Introduction

Fibroblast growth factors (FGF) are involved in the regulation of growth and regression of the corpus luteum (CL). A small FGF subfamily encodes non-secreted proteins known as FGF homologous factors (FHF 1 to 4) that are mostly expressed in the nervous system, where they bind to MAPK scaffold proteins and to voltage-gated sodium channels (1). Although FHF2 mRNA was detected in ovarian tumors, it is not known if FHFs are expressed in the healthy ovary. We assessed FHF (1 to 4) mRNA expression at different luteal developmental stages and the localization of FHF2 in the bovine CL.

Materials and Methods

Bovine CLs were obtained from abattoir ovaries and classed into four stages of development [n = 10/group; 1 = corpus hemorragicum, 2 = developing CL, 3= mature/early functional luteolysis CL, and 4 = structural luteolysis; (2)]. Tissue samples were submitted to total RNA extraction, FHF and cyclophilin (CYC-A, used as an internal control) expression were examined by real time RT-PCR. Immunohistochemical analysis was performed with a polyclonal human FHF2 antibody (Abnova; 1:200). The effect of CL developmental stage on FHF gene expression was tested by ANOVA, followed by Tukey-Kramer HSD test.

Results and Discussion

FHF (1 to 4) mRNA expression was detected in all four developmental stages. FHF1 expression was found to be developmentally regulated, being lower in stage 3 than in stage 1, whereas intermediate levels were observed in stages 2 and 4 (Fig. 1). Lower FHF1 expression seems to be associated with the onset of functional luteolysis, but the involvement of FHF-1 in the mechanisms controlling luteolysis requires further investigation. The immunohistochemical analysis revealed the presence of FHF2 in large and small luteal cells in the bovine CL. In conclusion, the present data demonstrate that FHFs are expressed throughout the luteal lifespan, suggesting that they participate in the regulation of luteal development and luteolysis.

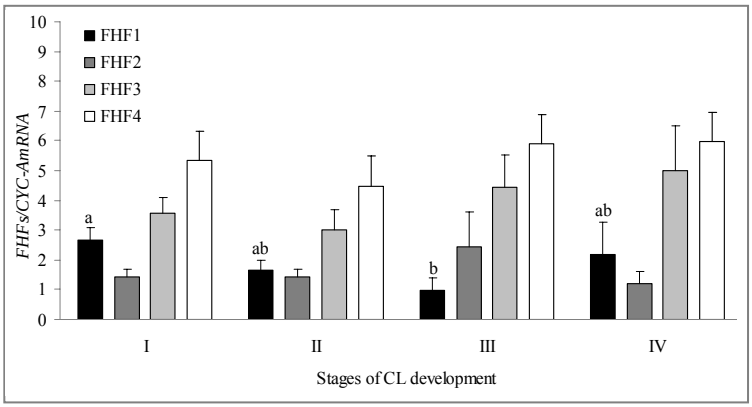

Figure 1. FHF1, 2, 3 and 4 mRNA expression in the CL development.

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Hypoxia, glucose uptake and vascularization in CL of non-pregnant dogs

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Introduction
Luteolysis in dogs, differently from farm animals, occurs independently of a uterine luteolysin (1). During canine functional luteolysis, a decrease in progesterone (P_4) concentrations occurs after day 20 after ovulation (Day 0 = ovulation). Morphological features of luteal and endothelial cells degeneration are first observed after day 45 after ovulation, concomitantly with a decrease in blood oxygen content in luteal tissue, which arises to hypoxic levels. Whether hypoxia plays a role in the regulation of corpus luteum (CL) function in the bitch is not clear. Angiogenesis and glycolysis are regulated by hypoxia through specific transcriptional factors up-regulation, notably hypoxia inducible factor-1α (HIF-1α, 2). Thus, we hypothesized that HIF-1α is involved in angiogenesis and glucose availability in different functional phases of canine ovarian cycle, especially during diestrus, contributing to local regulation of CL function.

Materials and Methods
For that purpose, we assessed mRNA levels of HIF-1α, Facilitative Glucose Transporter 1 (GLUT1), vascular endothelial growth factor (VEGF) and VEGF receptors - Flt-1 and KDR. Crossbred adult female dogs were submitted to ovarysalpingohysterectomy each ten days from day 10 to 70 after ovulation (n = 4/group). Blood samples were collected to P_4 assessment by RIA. The day of ovulation was considered the day when plasma P_4 levels reached ≥5ng/ml (3). CL were dissected and stored at – 80ºC until RNA was extracted, reversed transcribed and cDNA submitted to Real time (TaqMan) RT-PCR analysis. GAPDH was used as housekeeping gene. Statistical analysis was done using One-way analysis of variance followed by Newman-Keuls test. Correlations were done using Spearman's correlation test. Differences were considered significant when $p \leq 0.05$.

Results and Discussion
P_4 concentration was peaked at day 20 after ovulation (22.95 ± 3.84 ng/mL), and decreased continuously towards day 70 (0.67 ± 0.51 ng/mL). HIF-1α and GLUT1 mRNA expression was differentially regulated over diestrus showing a concomitant down regulation at day 40 after ovulation. and were positively correlated to each other ($r = 0.523$, $p = 0.01$). VEGF, Flt-1 and KDR mRNA expression was also cycle dependent and higher during the early and mid than in the late luteal phase. Although VEGF expression was highly positively correlated to VEGFRs expression ($r \geq 0.62$, $p \leq 0.007$), nor VEGF either VEGFRs showed any correlation to HIF-1α and GLUT1. P_4 concentrations were correlated to VEGF and VEGFRs expression ($r = 0.65$, $p < 0.05$), however, nor HIF-1α either GLUT1 showed any correlation to P_4. These findings suggest that HIF-1α acts directly stimulating GLUT-1 mRNA expression, apparently independent of P_4 production. Humoral factors, as P_4, may be involved in angiogenic process regulating VEGF expression (4) or vice-versa (5). Thus, our data point towards direct involvement of angiogenic factors in CL formation, lifespan and function. Additionally, our results provide evidence for the role of HIF-1α in glucose uptake by CL, suggesting that HIF-1α time-dependent expression is essential for luteal cells function maintenance under hypoxic stress.

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Evaluation of different protocols to induce cyclicity in prepubertal beef heifers

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Introduction

Progestogen treatment prior to ovulation leads to the normal duration of corpora luteum (CL) formed following ovulation (1). In multiparous postpartum anestrous beef cows, the incidence of estrus and onset of estrous cyclicity (ovulation and subsequent formation of a CL of normal life span) is increased following CIDR removal (2). This study was designed to evaluate the effect of the treatment with progesterone (P4) associated to the administration of estradiol cypionate (EC) on the device withdrawal on induction of cyclicity in prepubertal crossbreed (*Bos indicus x Bos taurus*) heifers.

Material and Methods

In the present study, it was used two hundred and twenty six non-cyclic heifers (absence of corpus luteum). Ultrasonographic examination was performed on Day 0 and Day 18 to measure the uterine diameter. On Day 0, the animals were homogenously allocated in one of the three treatments (Control, P4 and P4EC). Heifers from Control group did not received intravaginal device. Groups P4 and ECP4 received an intravaginal device (CIDR®, Pfizer Saúde Animal, Brazil) previously used for 24 days. On Day 8, the implant was removed (P4 and P4EC groups) and administered 0.5 mg of estradiol cypionate (ECP®, Pfizer Saúde Animal, Brazil) on P4EC group.

Results and Discussion

The treatment with progesterone increased the uterine diameter of heifers (Fig. 1 and Table 1). The results for Control, P4 and P4EC groups were respectively: cyclicity rate (presence CL on D18) = 18.7% (14/75)^{b}, 44.0 % (33/75)^{a} and 48.0% (36/75)^{a}. These results are in agreement with other report (2) that induced cyclicity in primiparous and multiparous beef cattle pre-treated with P4 intravaginal device. Additionally, other authors (3) working with anestrous beef cows found an increased of 44% on ovulation rate after treatment with P4. Present results show that the pre-treatment with progesterone intravaginal device is capable of inducing estrus cyclicity in prepubertal crossbreed heifers and increasing the uterine diameter.

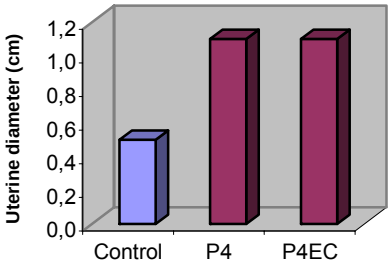

Figure 1. Effect of the treatment with progesterone on increase of uterine diameter of crossbreed heifers.

Table 1. Effect of the treatment with progesterone on the uterine diameter of heifers.

	Uterine Diameter (mm)		*P*
	D0	D18	
Control	13.4±0.7	13.9±0.3	0.53
P4	13.0±0.3^{b}	14.1±0.3^{a}	0.003
P4EC	13.3±0.2^{b}	14.4±0.3^{a}	0.003

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Influence of supplementation with protected unsaturated fat in pre-pubertal Nelore heifers on the ovulation rate, blood glucose and cholesterol concentration during the rainy season

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Introduction

Mammalian reproduction is influenced by many factors as species, breed, age, body condition score (BCS) and nutrition. Among these, nutrition has an important role because of its direct effects on physiology and reproductive performance. In bovine females, the amount of food intake or the food energy source affects many estrous cycle characteristics, such as cycle length, follicular wave patterns and size of ovarian structures. Food restriction can influence some of these variables because of the resulting reduction of blood glucose concentration. Also, the estrus length and intensity can undergo alterations depending on the quality of the food taken in (1). Intake of diets containing high levels of unsaturated fatty acids leads to an increase in blood cholesterol concentration and positively modulates the ovarian physiology of cows (2). The aims of this work were to evaluate the ovulation rate and the serum glucose and cholesterol concentrations in heifers receiving different types of diet.

Materials and Methods

Nelore heifers (n = 84) were maintained in Marandu pastures (*Brachiaria brizantha* cv.) since the age of eight months. Heifers were divided to graze at two different grass heights (15 and 35 cm) and to receive three supplementations strategies (two groups received Megalac-E® Protected Fat and the Control group received a mineral supplement in the diet). Blood samples were collected by puncture of the jugular vein in intervals of 28 days during the rainy season (January to May, 2008). Serum glucose and cholesterol levels were analyzed using commercials kits (Sigma). The ovarian structures (follicles and corpus luteum) were examined by rectal palpation for the detection of ovulations.

Results and Discussion

No cows grazing 15 cm grass in the Control Group ovulated (Table 1). In the remaining groups (supplemented with Megalac-E® Protected Fat), CL were founded precociously ($P < 0.05$). Throughout the experimental period, glucose levels were reduced ($P < 0.05$) in females maintained in the 15 cm pastures and remained constant in the female maintained in the higher pastures (height of 35 cm). The glucose levels were not affected by the supplementation ($P > 0.05$). The cholesterol levels remained constant in the control groups (142.2mg/dl in 15cm and 135.0 mg/dl in 35cm) but increased towards the end of the experimental period in the supplemented groups (202.7 mg/dl in 15cm and 200.7 mg/dl in 35cm). Nevertheless no significant difference was found in the cholesterol levels among the supplementation groups and the different heights of pasture ($P > 0.05$). The groups supplemented with Megalac-E® Protected Fat demonstrated precocious CL presence. Therefore, it can be concluded that supplementation is favorable to animal reproductive precocity.

Table 1. Corpus Luteum detection in the pre-pubertal Nelore heifers managed with different heights of pasture and supplementation.

Supplementation Groups	Corpus Luteum (average± SD)	
	15 cm pasture height	35 cm pasture height
Mineral Salt (Control Group)	$0.00 \pm 0.00^{b\,B}$	$0.21 \pm 0.43^{a\,A}$
Supplement 1	$0.14 \pm 0.36^{a\,A}$	$0.15 \pm 0.38^{a\,A}$
Supplement 2	$0.35 \pm 0.50^{a\,A}$	$0.21 \pm 0.43^{a\,A}$

Means with different superscripts in a row (minor letter) or in a column (capital letter) are different ($P<0.05$). N = 14/group.
Supplement 1: Citric Pulp, Cotton Bran, Megalac-E®, Minerals and Urea (26% CP and 81% TDN).
Supplement 2: Citric Pulp, Corn Gluten, Megalac-E®, Minerals and Urea (26% CP and 81% TDN).

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Timing of post-ovulatory progesterone rise and luteolysis in Colombian Sanmartinero cows

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Introduction

Importance of luteal function during early pregnancy has been well established (1). A delayed rise in post-ovulatory progesterone is associated with poor embryo development. Colombian native breeds of cattle have shown adaptation to the environmental conditions in tropics, in terms of reproductive performance (2,3,4). The aim of this study was to describe the post-ovulatory progesterone rise and its decrease after luteolysis during the estrous cycle in Sanmartinero cows.

Materials and Methods

15 suckling cows were studied during consecutive estrous cycles after postpartum onset of cycling. Blood samples were taken three times a week and progesterone levels were measured by radioimmunoassay.

Results and Discussion

Estrous cycle duration was 20.95 ± 1.99 days, with maximum progesterone levels of 10.23 ± 2.45 ng/ml on day 15 (Fig. 1). Basal progesterone levels under 0.5 ng/ml were found until day 3 post-ovulation, as found in other Colombian native breeds (2,3,4), after day 3 post-ovulation progesterone steadily increases at a constant rate until reaching its maximum level. Percentage of variation on progesterone levels between each sampling day regarding the previous one are shown on Fig. 2. Suprabasal progesterone levels were seen in connection with extended growth of preovulatory follicle in repeat-breeder animals (5). On the other hand, a high decrease on progesterone levels (49.44%) was presented between day 17 and 19, when luteolysis occurs. There is evidence that increased time from luteolysis to ovulation is associated with delayed progesterone increases after ovulation (1).

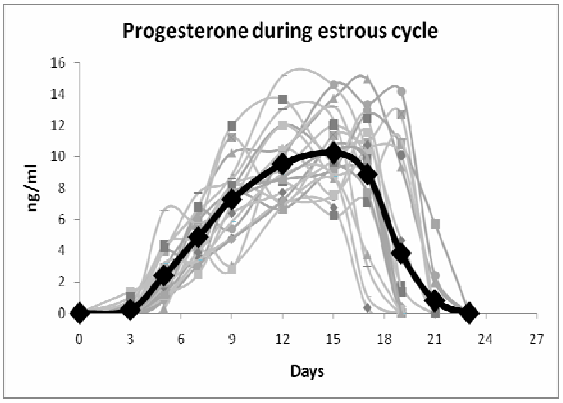

Figure 1. Average progesterone levels

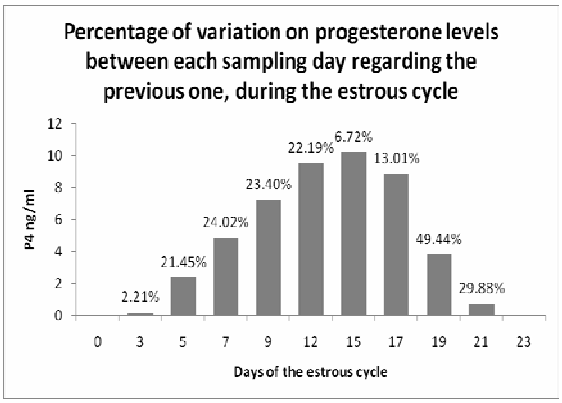

Figure 2. Percentage of variation on progesterone levels.

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Evaluation of luteolytic effects of Sodic cloprostenol (Sincrocio®) in Mangalarga (*Equus cabalus*) mares treated in different days of estrous cycle

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Introduction

The induction of ovulation in mares is a practice to optimize the reproductive management and improve the use of stallions. Thus, the objective of the present study was to evaluate the luteolytic effect of Sodic cloprostenol (Sincrocio®) administered in different days of estrous cycle to induce the heat in cyclic mares.

Material and Methods

In the present study, it was used 53 cyclic mares showing good body condition. The animals that presented follicles >35mm were treated with 2500IU of hCG (Chorulon®, Intervet, Netherlands) to induce ovulation. Only the mares that ovulated in the same day (Day 0; n = 29) were used at this study. The animals were allocated in one of three experimental groups [Day 3 (n=9), Day 7 (n=10) and Day 12 (n=10)]. Animals from each group received 250 µg of Cloprostenol (PGF) i.m. in different days of the estrous cycle (Day 3, Day 7 and Day 12. Blood samples were collected immediately before and after the PGF administration (0, 12, 24, 36 and 48 h). The samples were cooled, centrifuged (3000xg/5minutes) and then frozen to measure plasma progesterone concentrations by radioimmunoassay (Coat-A-Count Progesterone® (Diagnostic Products Corporation, Los Angeles, EUA).

Results and Discussion

The plasma progesterone concentration was significantly different before the administration of PGF on Day 3 compared to Day 7 and Day 12. The P4 concentration did not differ between treatment performed on Day 7 and Day 12 in any moment of the blood collection and decreased until 48 h after the administration. The P4 concentration was similar between all groups only in 12 and 24 h after the treatment. The P4 concentration of Group Day 3 decreased lesser (36 and 48 h) than Day 7 and Day 12 groups. According to these results, we can conclude that 250 µg of Sodic cloprostenol (Sincrocio®) was efficient to induce luteolysis when the treatment was performed on Day 7 and Day 12 after ovulation.

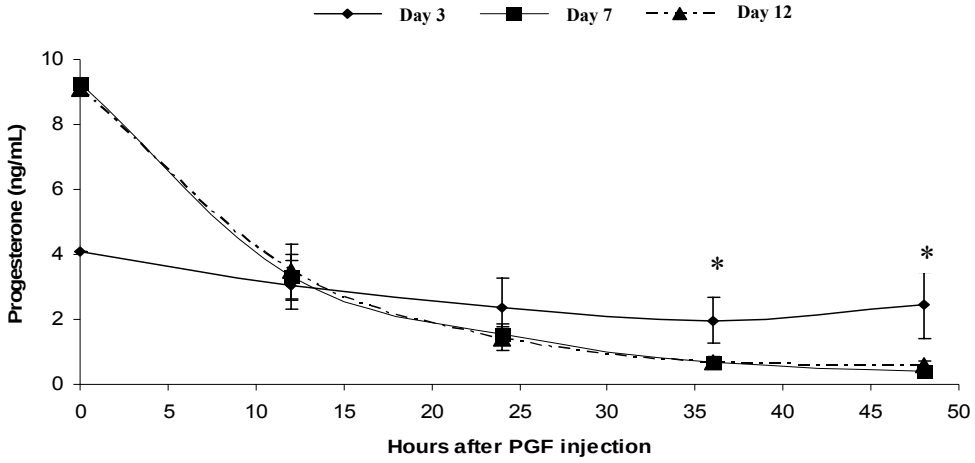

Figure 1. Plasma progesterone concentration immediately before and after (0, 12, 24, 36 and 48 h) the PGF administration 3, 7 and 12 days after ovulation (* Day 3≠Day 7 e Day 12, P<0.05).

Acknowledgments: Ouro Fino Saúde Animal and Central Fleury Reprodução Eqüina.

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Cellular changes in the uterine tube epithelium of the mare

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Introduction

The uterine tube is a key organ of the reproductive tract where major biologic events occur. It is documented that the epithelium of the uterine tube experiments changes throughout the reproductive phases in many species. In the cow, marked cyclic changes were described on the surface of the epithelium in the fimbriae and ampulla mainly, and fewer changes were found in the isthmus and uterotubal junction (1). Similar changes have also been described in the pig (2), the bitch (3) among other species. Briefly the main changes occur at the height of the cells, the proportion of ciliated and non ciliated cells, and the presence of secretory granules. One particular and interesting finding consists in protrusion of cytoplasmic extension towards the oviductal lumen; sometimes large cell fragments including nuclei and whole cells are also observed being extruded from the epithelium. Particularly in the mare, information available on cellular changes of the oviductal epithelium is very limited. Therefore, the objectives of this study were to investigate morphological changes in the oviductal epithelium of the mare at different reproductive stages.

Materials and Methods

Sixteen non-pregnant mares, aged 2-14 years, were selected at a local slaughterhouse plant and assigned to be sacrificed at different times. Reproductive status was determined by rectal palpation and ultrasonography. Four mares were selected at the following stages: a) anovulatory phase, with follicles less than 15 mm, b) estrus, with follicles of 35 mm or larger and uterine oedema, c) diestrus, at day 7-8 post ovulation, and d) postovulatory mares, at day 1-2 post ovulation. Reproductive tracts were collected immediately after the mare´s death and placed in ice. The oviduct was freed of connective tissue and samples from the ampulla, ampullary-isthmic junction (a-i-j) and isthmus were taken for histopathology (Hematoxylin/Eosin) and stained also with Hoescht 33258 for identification of nuclei by fluorescent microscopy.

Results and Discussion

Marked histological changes in the epithelium of the uterine tube were observed associated with the reproductive stages. Ciliated and nonciliated cells were present in the three regions at all stages examined, with ciliated cells constituting the major cell type. Cell height varied among the stages being shorter at the anovulatory phase. At diestrus, the epithelial cells showed a very consistent location of the nuclei at the base of the cells. In contrasts, in estrus and much more at postovulation, the nuclei were found at different locations within cells, many toward the apical surface or even protruding into the lumen. Some times whole cells were found like being “pushed or eliminated” to the lumen, this was mostly observed in the ampulla and the a-i-j. The morphological appearance of these cells was not apoptotic but would indicate some kind of renewal process. This study confirms for first time that in the mare, epithelial extrusion of large cell fragments including nuclei and whole cells also occurs. The mechanism of this process of cell elimination seems to vary among species. Non-apoptotic cell loss of non-ciliated cells occurs in large (pig, sheep, goat, cattle) and small animals (dog; 4). However, apoptosis was commonly observed in many areas of the cat oviduct epithelium.

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Apoptosis changes in the ovaries of rats submitted to feed restriction during embryonic development

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Introduction

Delayed ovary development during the embryonic period has been shown to result from mal-nutrition during pregnancy. As a result there is a decrease in pre-antral follicles formation that can lead to fertility problems at adulthood (1), such as changes in follicular atresia and the expression of apoptosis genes regulators, which are an important component of embryonic development and follicular function until later in life (2). The Bcl-2 gene family members are involved in cell proliferation, follicular selection and luteolysis, and the ratio between Bcl-2 (pro-survival molecule)/BAX (pro-apoptotic molecule) shows to be critical for the normal survival of germ cells (the higher the ratio the lower the apoptosis). This study aimed to establish the relative expression of apoptosis genes mRNA in the ovaries of rats whose mothers were submitted to feed restriction during pregnancy and verify the efficacy of early dietetic correction after parturition on such expression.

Materials and Methods

During pregnancy 24 female Wistar rat (*Rattus novergicus*) were divided into two groups: GI, received free-feeding diet; GII received 70% of normal daily consumption. GI was considered the control group and GII experimentally produced the malnutrition during pregnancy. After parturition, during lactation, the GII females received free-feeding diet similar to GI, to establish the dietetic correction. The offspring ovaries were submitted to histological analysis (embedded in resin and stained with hematoxilin-eosin) and quantification of relative mRNA abundance for Bcl-2 and BAX (RT-PCR) at birth, weaning and puberty.

Results and Discussion

No difference in the number of oocytes/mm^2 (18 x 10^3) between groups at birth ($p > 0.05$). There was a difference in the proportion of pre-antral follicle (GI = 26.17%; GII = 44.07%, $p = 0.053$) and large-antral follicles (GI = 26.12%; GII = 16.67%, $p=0.028$) between treatments at weaning. On the other hand, the proportion of small-antral follicles was the same between GI and II (GI = 47.71%; GII = 39.27%, $p = 0.923$). At puberty, the animals did not present differences ($p > 0.05$) in the different follicle categories (pre-antral follicles = 37.17%, small-antral = 29.62%, large-antral = 33.23%). Regarding the relative gene quantification of Bcl-2 and BAX, a difference between experimental groups was observed only at weaning (Fig. 1), with GI showing higher relative expression of apoptotic genes (GI = 2.26 ± 0.82; GII = 0.51 ± 0.30, $p = 0.024$). Animals from GI presented a lower Bcl-2/BAX ratio at birth (suggesting higher apoptosis), that tended to increase and stabilize from weaning. On the other hand, GII at weaning presented a lower Bcl-2/BAX ratio that increased to normal values at puberty. It is suggested that higher expression of BAX from GII weaning animals could occur due to the inferior number of viable follicles (2). Feed restriction during pregnancy was sufficient to cause an unbalance on follicular population and on expression of genes related to apoptosis. Finally, the early dietetic correction (from birth) reverted the alterations when the animals reached puberty.

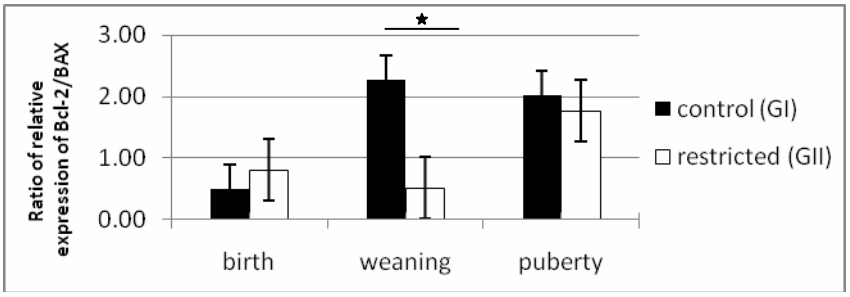

Figure 1. Ratio of relative expression of Bcl-2/BAX of control (GI) and feed restricted (GII) groups at birth, weaning and puberty.

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Immunolocalization of proteins involved in the signal transduction mechanism of p-ERK ½ in canine prostates with different diagnoses of Benign Prostate Hyperplasia

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Introduction

The canine prostate gland is the only accessory gland of the genital apparatus of this species and it is a locus of several pathological processes in middle-aged to senile animals (1), such as Benign Prostate Hyperplasia (BPH). The proliferation, differentiation, and organization of animal tissue cells are controlled by various forms of intercellular communication. One of these forms involves a network of polypeptides such as growth factors, which activate membrane receptors for the transduction of proliferation and differentiation signals (2). The aim of this study consisted in immunolocating the proteins *CYR61*, EGF, TGF-α, EGFr, and p-ERK1/2 via immunohistochemistry (IHC) in canine prostates with by different BPH diagnoses.

Material and Methods

Twenty-three prostate fragments were investigated, of which 5 were considered normal, 6 were diagnosed with benign prostate hyperplasia of the epithelial type (ep-BPH), 6 with benign prostate hyperplasia of the stromal type (s-BPH), and 6 with complex benign prostate hyperplasia (CBPH). All fragments were assessed by the IHC technique (3) for the polyclonal antibodies (and respective concentrations) anti-*CYR61* (1/400), anti-EGF (1/20), and anti-p-ERK1/2 (1/200) as well as for the monoclonal antibodies anti-TGF-α (1/20) and anti-EGFr (1/20). Secondary polyclonal and monoclonal biotinilate antibodies (Vectastain Elite Kit ABC – Vector laboratories, Burlingame, CA, USA) followed the concentration pattern indicated by the manufacturer. Lamina treated with polyclonal and monoclonal antibodies were assessed under optical microscopy with regards to the presence and absence of marking in stromal components (str. c.) - smooth muscle fibers (SMF), fibroblasts (F), smooth musculature of arteries and/or arterioles (SMA), endothelial cells (EC), and mononuclear cell infiltrate (MC) - and epithelial acini (epith. c.), such as cytoplasm and nucleus of secretory cells.

Results and Discussion

All investigated fragments (n = 23) revealed a positive marker for *CYR61*, EGF, TGF-α, EGFr, and p-ERK1/2 proteins. Stromal and epithelial acini components also revealed a positive marker in various levels of intensity. Only the normal fragments failed to show a significant marker in stromal components for TGF-α and EGFr proteins (Table 1).

Table 1. Stromal and epithelial components which revealed the most prominent protein markers in each diagnosis of canine prostate under study.

Protein	Diagnoses							
	Normal		Ep-BPH		S-BPH		CBPH	
	Str.C	Epith.C.	Str.C.	Epith.C.	Str.C.	Epith.C.	Str.C	Epith.C.
CYR61	SMF, SMA	nucleus	SMF, SMA	nucleus	SMF, SMA	cytoplasm	SMF, F	nucleus
EGF	SMF, F	nucleus	SMF	nucleus	FML	nucleus	SMF, EC	nucleus
TGF-α	X	cytoplasm	SMF, MC	cytoplasm	MC, SMF, F	cytoplasm	EC	cytoplasm
EGFr	X	nucleus	SMF, SMA	nucleus	SMF, F	nucleus	SMF, F	nucleus
p-ERK 1/2	SMF, F	nucleus	SMF	nucleus	SMF, F	nucleus	SMF, EC	nucleus

Research on proteins involved in cellular differentiation and proliferation has been applied to several tissue alterations within veterinary medicine. The comparison of the immunolocalization of these proteins in both normal and altered tissues is of vital importance for the knowledge of their actions, for the establishment of signal transduction routes, and for the production of data applied to pharmacological studies in order to block the superexpression and/or activation of such proteins and thus reduce chances of neoformations of altered tissues.

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Identification of IGF-II from mRNA in Boer bucks spermatozoa

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Introduction

Evaluation of protein pattern in spermatozoa is a crucial step to identify their role in the molecular regulation of reproductive mechanisms. In humans (1), cattle (2) and other species (3), IGF-II has been identified in spermatozoa and was positively correlated with semen quality. Thus, the aim of this study was to identify the IGF-II in the Boer bucks spermatozoa.

Materials and Methods

Fifty semen samples were collected from six adult Boer bucks with normal seminal parameters according to (4). Spermatozoa were separated from seminal plasma by centrifugation (15000 rpm/15') and stored in -20 °C until RNA extraction. In twenty-five samples, spermatozoa were capacitated with swim-up method according to (5). The total RNA extraction was initiated transferring the pellet to a new tube, in which was added Trizol, and, subsequently, the mixture was incubated for 5 minutes at room temperature. After digestion, aqueous phase was extracted with chloroform and the RNA precipitation was performed using isopropilic alcohol in a proportion of 1:1. The pellet was washed with ethanol 75%, dried at ambient temperature for 15 minutes, resuspended in DEPC water and stored at – 80 °C. The total RNA samples were treated with DNAse. To detect IGF-II in spermatozoa mRNA, RT-PCR and electrophoresis in gel-agarosis was performed in agreement with (6).

Results and Discussion

The primers used were based on the bovine sequence of IGF-II gene (P07456). Amplifications procedure showed a product of 63,5 bp (Fig 1). The presence of IGF-II was verified respectively, in each buck, in the capacitated spermatozoa and in the spermatic pool (Fig 1). Also, from all spermatic RNA samples, it was isolated a PCR product with similar molecular weight. These results are in agreement with the studies carried out in humans (6, 7). In conclusion, our findings showed that IGF-II is present both in non-capacitated sperm (individually or in pool), as the capacitated buck sperm.

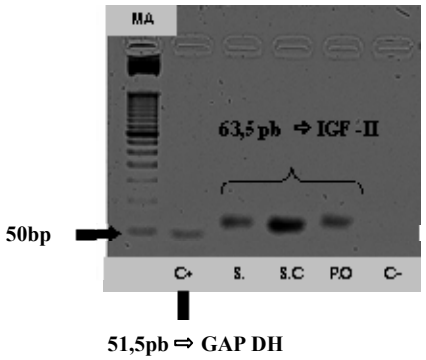

Figure 1. RT-PCR of IGF-II in the goat spermatozoa. Well 1(MA): molecular marker of 50bp, Well 2 (C+): positive control – GAPDH, Well 3(S.I): semen sample for individual , Well 4(S.C): capacitated spermatozoa, Well 5(P.O): sperm pool, Well 6(C-): negative control.

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Apoptosis in embryos produced by fertilization or chemical activation from heat-stressed bovine oocytes

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Introduction

Heat stress compromises oocyte viability, affecting further development of in vitro fertilized or chemically activated bovine embryos [1]. Moreover, embryos exposed to heat stress have an increase in apoptotic index [2]. The aim of this study was to evaluate the effect of heat stress during oocyte maturation on cell number and apoptosis in blastocysts produced by in vitro fertilization or chemical activation.

Materials and Methods

Cumulus-oocyte complexes were distributed in two groups: in vitro maturation at 38.5ºC for 24 h (control group) or in vitro maturation at 41ºC for 12 h followed by 38.5ºC for 12 h (stressed group). After maturation oocytes were in vitro fertilized with sperm from Holstein bulls or parthenogenetically activated with ionomcyn and 6-DMAP, according to the following treatments: (1) parthenogenetically activated oocytes from control group; (2) parthenogenetically activated oocytes from stressed group; (3) in vitro fertilized oocytes from control group; and (4) in vitro fertilized oocytes from stressed group. Zygotes were cultured in CR2aa medium. The embryos in eighth day were fixed and permeabilized for TUNEL assay (DeadEnd^{TM} Florimetric TUNEL System-PROMEGA), according to the manufacturer instructions. Total cell number, apoptotic cell number and apoptotic cell index (calculated by dividing the apoptotic cell number by total cell number) were analyzed by analysis of variance and mean compared by Student Newman Keus. Significance was estimated at the level of P = 0.05.

Results and Discussion

In vitro fertilized blastocysts had higher ($P < 0.01$) total cell number and lower ($P<0.01$) apoptotic cell index than parthenogenetic blastocysts (Table 1). Total cell number and apoptotic cell index was similar ($P>0.05$) for blastocysts produced from stressed and non-stressed in vitro fertilized oocytes as well as for blastocysts produced from stressed and non-stressed parthenogenetically activated oocytes (Table 1). This result is similar to previous study [3] with in vitro fertilized bovine embryos but it is different from study [4] with parthenogenetic swine embryos. In conclusion, this study suggests that heat stress during first 12 h of in vitro oocyte maturation does not interfere on apoptotic process in blastocysts, regardless of in vitro fertilization or chemical activation.

Table 1. Total cell number and apoptotic cell index (mean ± SE) in blastocysts produced from in vitro fertilized or chemically activated heat stressed-oocytes.

Treatment	No.	Total cell no.	Apoptotic cell no.	Apoptotic cell index
Non-stressed and parthenogenesis	27	78.51 ± 8.10 ^{b}	19.70 ± 2.59 ^{a}	0.26 ± 0.02 ^{ab}
Stress and parthenogenesis	25	80.56 ± 8.63 ^{b}	22.60 ± 2.32 ^{a}	0.30 ± 0.02 ^{b}
Non-stressed and fertilization	27	128.38 ± 9.18 ^{a}	25.46 ± 3.15 ^{a}	0.19 ± 0.01 ^{a}
Stressed and fertilization	18	108.72 ± 9.04 ^{a}	21.72 ± 3.00 ^{a}	0.19 ± 0.02 ^{a}

Means with different superscripts within a column differ at $P<0.05$.

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Oct-4 and vimentin expression in *Agouti paca* embryos

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Introduction
Research involving stem cells showed that the protein OCT-4 is the most used as a marker of pluripotential mobile (1) and first described in embryonic cells of mice (2). The vimentin is a marker of mesenchyme of mesoderm embryonic cells and also used in embryos to colocalized as fibroblasts intermediary filament (3). The objective was to identify pluripotential of embryonic cells of *Agouti paca* through immunomarked with OCT-4 and vimentin.

Materials and Methods
They were used 2 embryos of *Agouti paca* with 1.8 cm of Crow-rump (CR) and another with 1.2 cm of CR, set in paraformaldeyde 4%, dehydrated and embedded in paraffin. Sequential slides of 5µm were carried out, followed by the processing for the immunohistochemistry.

Results and Discussion
The Oct-4 as a marker of pluripotential showed up in the heart positively to the embryo of 1.2 cm in CR in the region of myocardium, as well for the kidney in both embryos in the tubules region and, liver, as in a important body haematopoiesis in the hepatocytes cords. These findings corroborate to the results of literature, which cites that Oct-4 expressed in cattle is located in blastomere, in the internal cell mass of the blastocysts, in epiblast and embryonic stem cells (4), as well as lines of cells from human tissues of adult liver, heart and bone marrow (5). Also note that the same was not expressive in the lung, intestine and somites for the two embryos. The vimentin as a marker of mesenchyme cells and the embryonic mesoderm made up positive in both embryos, as in the lungs and bronchi in the region of capillaries that nourish the region of epithelial cells. In the heart, this marker was positive in micro-vessels, mesenchyme and heart. In the intestine mesenchyme around the intestinal tubules and blood capillaries proved to be positive for vimentin, but also in somites, which were found around the cartilage and mesenchyme. In the liver, regions found positive for vimentin were mesenchyme cells and the endothelium of sinusoids, while in the kidney, positive in mesenchyme and was seen in the region around the kidney tubules and the capillaries. As in our results, the term positive for vimentin mesenchyme of embryos in pigs (6) and human (7) is expressed in cells of epiblast and hypoblast, which brought the mesoderm and endoderm, respectively.

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Fused placentomes development in bovine placenta

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Introduction

For many years the placenta of animals has stimulated the curiosity among researchers. Currently many techniques are used to analyze its structure. Being a transitory organ, the placenta is very dynamic and mutable, being difficult to accurately measure, especially when considering groups of different gestational stages. Placentome formation begins with trophoblast growth and they show various fusion degrees (1, 2, 3). The current study has the objective to analyze the region of the placentome fusion during gestation and its cellular contribution to placental development among non-manipulated cows.

Material and Methods

28 uteri from non-manipulated bovine pregnancies were obtained from a slaughterhouse in Sao Jose dos Campos – SP. Samples of placentome fusions were collected and put under liquid nitrogen to determinate the cell cycle stage through flow cytometry. These cells were separate in pH 7.6 citrate buffer and processed according to the (4) technique.

Results and Discussion

As seen in Fig. 1, the placentome fusion showed a higher number of cells in proliferative activity (G2/M phase) and apoptosis on the last gestational group (12.1% ± 9.0 and 11.4% ± 9.0) in comparison to group II (5.4% ± 3.4 and 3.5% ± 0.7). Both cellular proliferation and apoptosis have an important role in placental function and are inversely proportional during gestation (5). However, the placentomal fusion showed equilibrium in proliferative and apoptotic cell proportions throughout gestation, characteristic of connective tissue. According to (6) cell proliferation indicates that they are part of the concepts nutrition; being so, in a gestational moment when the fetus is already fully developed and duo to leave the uterine cavity, there is no physiologic reason in continuing the proliferative process. The equilibrium in proliferative and apoptotic cells found in placentomal fusions indicates that this region propitiates the maternal-fetal maintenance, but has no role in placental maturation and releasing.

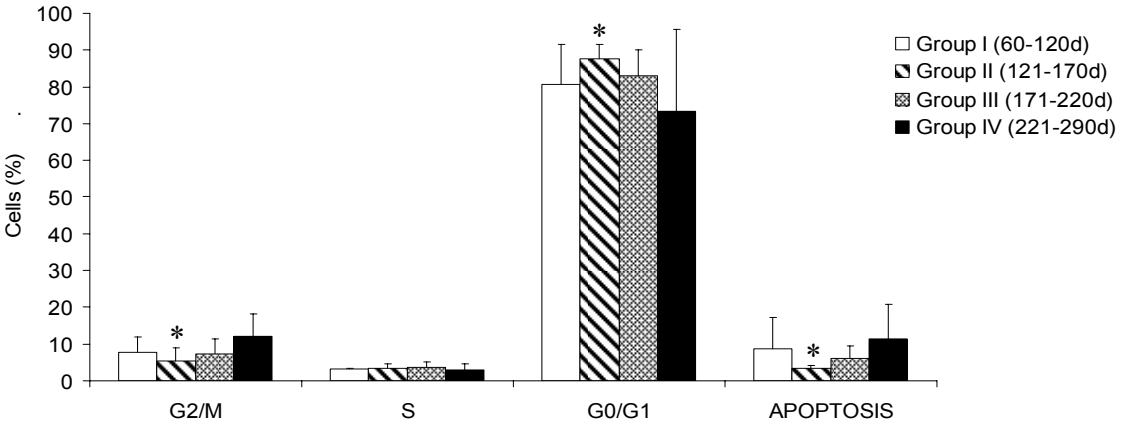

Figure 1. Placentome fusion cell distribution on cellular cycle stages, during gestation. *P<0.05: group II *vs.* group IV.

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Differential separation of the yolk sac proteins of not manipulated bovine embryos by Two-dimensional Electrophoresis (2DE)

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Introduction

The yolk sac is one of the membranes that perform an important role for the initial survival of the embryo in many mammalian species, besides to produce the necessary proteins for its development (1). Its function is complex, participating of hematopoiesis and maternal material transference, such as amino acids, vitamins and proteins, controlling the most of the cell processes that occur in a great diversity, acting like enzymes, antibodies, hormones, structural components and cell receptors (2). The large scale proteins study is known as proteomic, traditionally associated to an exposition of a great number of proteins of a cell line or organism on two-dimensional polyacrilamide gels (3, 4, 5). In this experiment, we aimed to study the establishment of a proteins extraction protocol of the bovine embryos yolk sac adequate to a proteomic analysis of this membrane, which will be determinant for the development of future projects.

Material and Methods

The protocol developed in this article presented results with a high resolution of the spots on electrophoresis two-dimensional (2-DE) gels (6). The protocol was constituted by the following steps: (i) grinding in liquid nitrogen, (ii) followed by tissue washing in Tris-Cl buffer and (iii) in solution of protease inhibitors. Next, the samples were lysed in re-hydration buffer and separated by Isoelectric Focalization in first dimension and by polyacrilamide gel electrophoresis for second dimension. The gels were dyed with Coomassie blue G-250, scanned in the Image Scanner and the images were analyzed with Image Master 2D Platinum 6.0 software.

Results and Discussion

Using this protocol, the final preparation of proteins presented itself in solution and it was observed well-defined patters of 2-DE in a pH range of 3-10. We compared our results to a researchers group that have worked with rats embryos yolk sac (7), which demonstrates as well a high reproducibility of the proteins spots already described and new groups of not known proteins and that possibly present a fundamental role in differentiation of the yolk embryonic cells and of other appendages during different stages of a normal bovine gestation.

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Aromatase gene expression in intact and castrated Syrian hamster females

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Introduction

In premenopausal women, the ovaries are the principle source of estradiol, however, in postmenopausal women estradiol is produced in a number of extragonadal sites. We hypothesize that CYP19 gene, responsible for aromatase (ARO) synthesis, is expressed in several non ovarian tissues of female Syrian hamster, especially after ovariectomy. Real time PCR was used to verify the expression of aromatase mRNA in these tissue comparing intact (IN) and ovariectomized (OX) females.

Material and Methods

Four out of eight heterogenic, adult (age, 10wk), sexually mature female Syrian hamsters were ovariectomized. Three months later all the animals were euthanized and tissue samples (ovary [IN], adrenal, brain, adipose, cartilage, aorta, liver, small gut and lungs [IN and OX]) were collected from both intact and castrated females. Tissues had their total RNA isolated and RT-PCR was performed to synthesize cDNA. Real time PCR was carried out using Platinum SYBR Greener qPCR SuperMix (Invitrogen, Carlsbad, CA, USA) in a thermal cycling profile of 40 cycles of 15s at 95 ºC and 45s at 60 ºC for ACTH amplification (used as housekeeping gene) and 40 cycles of 15s at 95 ºC and 45s at 61 ºC for ARO amplification. Primer sequences were AROf 5´ GGCCTCCTTGTCTTGATTTG 3' and AROr 5´ CAATCCCCATCCACAAGAAC 3´ for ARO amplification and ACTHf 5' AGGACTCGTACGTGGGTGAC 3' and ACTHr 5´ AGGTGTGGTGCCAGATCTTC 3', for ACTH amplification. Quantitative PCR data were analyzed using REST MCS beta software by Pair Wise Fixed Reallocation Randomization Test. Reactions were normalized by expression of housekeeping gene ACTH.

Results and Discussion

ARO mRNA expression was not detected in adipose, cartilage, aorta, liver, small gut and lungs. As expected, a notable expression of ARO mRNA occured in ovaries. However, the main finding of the study was that ovariectomy resulted in a significant increase of ARO expression in brain and adrenal ($p < 0.05$).

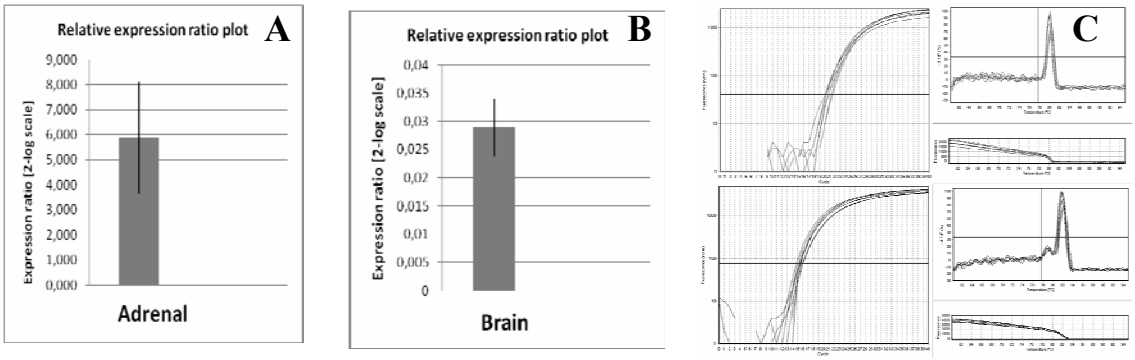

Figure 1. Changes in ARO mRNA expression in IN and OX females by real time PCR. ARO mRNA expression was normalized with ACTH mRNA expression. ARO expression was upregulated in OX group in adrenal (A) and brain (B) when compared to IN ($p<0.05$). (C) Amplification and dissociation curves for ARO and ACTH.

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Isolation, culture and characterization of Committed Hepatic Stem Cells for differentiation studies

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Introduction

Transplant therapy is normally limited by available organ donors. One of the goals of Regenerative Medicine is to find sources of cells and tissues for replacement therapies. Embryonic stem cells are a promising source of transplantable material due to the unique capacity of indefinite expansion in culture (primary cells), being a renewable source of tissue. Bipotential cells from the hepatic bud (HB) have the potential to be differentiated into hepatocytes, to be used for bridge transplant to treat hepatic failure (1). However, it is necessary to isolate the cells with higher proliferation and differentiation potential, as well as to develop efficient techniques of isolation and differentiation for these cells for successful of cell therapy. Thus, this work aims to isolate, culture, and characterize HB cells with increased differentiation potential to be used in cell therapy protocols.

Material and Methods

Embryos of isogenic rats (Fischer 344) with a gestational age between E11.5 to E16.5 were collected and measured using the Crow-Rump (CR) method. The HB (or the liver itself in the older embryos) was isolated from the embryos by microsurgery, under a stereoscope. The fragments were submitted to mechanic fragmentation, and the explants were cultured (DMEM-High glucose supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in 37°C, 5% CO2 under absolute humidity) on 3.5cm Petri plates. Embryos were fixed with Paraformoldehyde 4% (P4%) or Metacarn (Met). Immunohistochemistry for Oct-4 (1:100), Vimentin (1:300), Albumin (1:100), and PCNA (1:200) was carried out.

Results

Embryos fixed in P4% exhibited 32.9% more retraction in CR size than those fixed in Met. In E11.5 (CR: 3.5mm) the HB could not be isolated. On day E12.5 (CR: 7.23±0.16mm) the HB, composed by 2 segments, was found right below the heart. On day E14.5 (CR: 12.34±1.30mm) livers presenting 4 lobes, were found. Explants cultured from E13.5 (CR: 9.30±0.43mm), released oval shaped cells after 1d, which attached to the plate, and presented a fibroblast-like cell shape after 2d. Culture of E12.5 explants exhibited attachment and the fibroblast-like cell shape already on the first day of seeding. The cells in culture presented morphologic differences between the embryonic stages, being bigger when obtained on day E12.5 compared to the other gestational periods analyzed. Immunohistochemistry revealed an increased positive reaction for Oct-4, PCNA, and Vimentin in E12.5 samples than in other gestational periods. Albumin positivity was not identified until E16.5.

Discussion and Conclusions

Compared to P4%, Met is a better fixative solution for morphometric studies, as it causes minor retraction of the fixed tissue. From E12.5 on, the hepatoblasts present developed rough endoplasmic reticulum, possibly enabling these hepatoblasts to release proteins (2). However, there was no positivity for albumin in the gestational period studied. The results obtained, until this moment, allow us to propose that the bipotential cells from HB of F344 rats, obtained on day E12.5 should be preferably indicated for isolation, culture and the following differentiation into functional hepatocytes. This proposal is based on the cellular multipotentiality and enhanced proliferation, as evidenced by the strong positivity for PCNA, higher attachment speed, and *in vitro* proliferation.

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Testis structure, duration of spermatogenesis, and Sertoli cell and spermatogenic efficiencies in several different dog (*Canis familiaris*) breeds

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Introduction

Testis structure and organization are very similar in mammals. However, each species might eventually present particular morphofunctional characteristics that are related for instance to its reproductive strategy and/or behaviour. As an illustration of this aspect we could mention that the duration of spermatogenesis, that is controlled by the germ cell genotype, takes approximately 30 to 78 days in most mammals already investigated (1). Also, Sertoli cell efficiency that directly reflects on the daily sperm production per testis (DSP/g/T or spermatogenic efficiency) shows great variation among different mammalian species (2). Although there are hundreds of dog breeds, our knowledge regarding their testis function is still very scarce. The present study is part of a broader investigation, in which testicular parameters of ten different dog breeds (small, medium, standard and large) are being evaluated. In this regard, in this work our main goal was to comparatively investigate several important qualitative and quantitative testis parameters related to spermatogenesis and Leydig cells in six different breeds.

Materials and Methods

Sexually mature dogs from different breeds (mongrel dogs, n = 10; pincher, n = 12; beagle, n = 5; American pit bull, n = 9; poodle n = 12; and German shepherd, n = 6) were utilized. Intratesticular injections of tritiated thymidine were performed in order to determine the duration of spermatogenesis, and the animals were orchiectomized at different time periods after injection (approximately 1 hour, 2 and 3 weeks). The testes were fixed by immersion in 4% buffered glutaraldehyde and the obtained testis fragments were embedded in glycol methacrylate, stained with toluidine blue or PAS, and routinely prepared for histological and morphometrical evaluation.

Results and Discussion

Interestingly, an inverse correlation was observed between body weight and gonadosomatic index, suggesting that smaller breeds invest relatively more in reproduction. According to the acrosomic system, eight stages of the seminiferous epithelium cycle were characterized and their frequencies were very similar in the six dog breeds investigated. In comparison to pit bull (55.8 days), the duration of spermatogenesis was longer (61.7 days) in mongrel dogs, poodle, pincher and beagles. However, pit bulls presented the highest ($p < 0.05$) volume density (%) and number of Leydig cells per gram of testis. There was a tendency for inverse correlation between Sertoli cell number per gram of testis and Sertoli cell efficiency. Probably due to high seminiferous tubule volume density (%) and Sertoli cell efficiency, poodles showed the highest ($p < 0.05$) DSP/g/T in comparison to the other breeds investigated. The data already obtained indicated that some breeds (i.e. poodle) seem to have directed more energy to improve their sperm production. On the other hand, breeds like pincher and specially pit bull apparently invested more in the intertubular compartment, where androgens are produced. Taken together, the data already obtained reinforce that comparative studies are a powerful tool for a better understanding of reproductive biology, particularly in dogs that naturally present for instance a great variability in size and behaviour.

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Postnatal Leydig cells proliferation and the establishment of spermatogenesis is not a random event in pigs

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Introduction

During establishment of spermatogenesis at the prepubertal age, the seminiferous tubules maturation is first characterized by the formation of tubular lumen. This event is directly associated with Sertoli cell fluid secretion and it is considered the most important morphofunctional maturational marker for this key testicular somatic cell. In horses, in which the *rete testis* is excentrically located in the testis parenchyma, it is described that the maturation of the seminiferous tubules takes place initially in the central area of the testis (1). However, in most of the mammalian species already investigated, the seminiferous tubules maturation is considered to occur randomly throughout the testis (2). Ongoing studies from our laboratory related to postnatal Sertoli cell proliferation in pigs suggested that spermatogenesis is asynchronously established in the different regions of the testis parenchyma. In this regard, our main goal in the present study was to perform a more detailed investigation, during postnatal testis development in pigs, in three different regions of the testis parenchyma.

Materials and Methods

Twenty prepubertal (7, 30, 60, 90 and 120 days of age) and four postpubertal (180 days) crossbred pigs, from the Experimental Farm of the Veterinary School at the Federal University of Minas Gerais, were utilized. These animals were orchiectomized and testes tissue samples were obtained from three different regions of the testis parenchyma: a) near the tunica albuginea (TA); b) in the transitional area between the seminiferous tubules and the *rete testis* (TR); and c) the intermediate area (ID) between TA and TR. These samples were fixed by immersion in 5% buffered glutaraldehyde, embedded in glycol methacrylate, and routinely prepared for histological and morphometrical evaluation. All data are presented as the mean ± SEM. Student t-test and analysis of correlation were performed using STATISTICA 3.11 for Windows (StatSoft, Inc., Tulsa, OK). $P < 0.05$ was considered significant.

Results and Discussion

In all of ages investigated, it was observed that the seminiferous cords/tubules from TR and ID regions showed a tendency of higher diameter than those from TA region, mainly at 90 and 120 days of age. The tubular lumen was observed for the first time at 90 days, and the lumen volume density (%) at this particular age was 5 ± 4, 3 ± 1 and, 0.3 ± 0.2, respectively in TR, ID and TA regions. Considering that fluid secretion and Sertoli cell nucleus size are good markers of Sertoli cell maturation, we observed a significant and positive correlation between nuclear diameter and lumen formation (ID, r = 0.83; and TR, r = 0.47). No clear trend was observed for the pattern of Sertoli cells proliferation in the three different regions investigated. Nonetheless, the results suggest that seminiferous tubules maturation in pigs takes place initially in TR and ID regions, whereas in TA this process seems to occur at a later period. Remarkably, when the intertubular compartment was evaluated, striking differences were found for Leydig cells size. In all ages investigated, Leydig cells nuclear diameter and volume, and cytoplasmatic and cell volumes in ID were higher ($P < 0.05$) in comparison to the other two regions, whereas the mitotic index of these cells was lower in ID than in TR and TA, specifically at 7, 30 and 180 days of age ($P < 0.05$). Taken together, and similar to horses, the data herein found strongly indicates that functionally the testis development in pigs is an asynchronous event that might be related, at least at the beginning, to the interactions between Leydig and Sertoli cells. Moreover, the results obtained solely for Leydig cells suggest that these steroidogenic cells are more active in ID during the entire period investigated. Therefore, this particular event deserves a more carefull investigation and might explain, at least in part, why the data from experiments carried out in pigs, regarding for instance to Sertoli cells proliferation, are usually difficult to be interpreted.

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Stereological and structural investigation of the testis in inducible nitric oxide synthase (iNOS) adult knockout mice

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Introduction

Nitric oxide (NO) has been shown to play a crucial role in several physiological and pathological conditions. It is also well known that NO in mammalian tissues is produced from L-arginine via catalysis by NOS synthase (NOS) isoforms. NOS plays both beneficial and detrimental roles, depending on the activation status of cells (1). Both nNOS and eNOS are involved in normal physiological events that occur within the male reproductive system, including direct modulation of the biosynthesis and secretion of the steroid hormones by the Leydig cells in the testes. Excessive NO production, which is induced mainly by iNOS, may be associated with tissue damage. Nevertheless, although various NOS isoforms, including iNOS, eNOS and nNOS, are involved in the reproductive system physiology, little is known about the patterns of expression of NOS isoforms in the testes. In this regard, we are currently developing a study investigating several important parameters related to spermatogenesis and the testis structure in iNOS knockout mice. Particularly, we are presenting preliminary data already obtained for histological and stereological analyses of the testis and the frequencies of the different stages of the cycle of the seminiferous epithelium, characterized according to the acrosomic system.

Materials and Methods

Three knockout (KO) adult iNOS male mice on the pure genetic background and twelve wild type adult (70 days of age) C57BL/6 mice were utilized. After being perfused-fixed trough the left ventricle with 4% glutaraldehyde these animals had their testis dissected and weighed. Testis tissue fragments were embedded in plastic (glycol methacrylate) and routinely prepared and stained with blue toluidine for histological and morphometric analyses. All data are presented as the mean ±SEM. Student t-test was performed using STATISTICA 3.11 for Windows (Stat Soft, Inc., Tulsa, OK). $P<0.05$ was considered significant.

Results and Discussion

The body weight in the control and KO mice were similar ($p>0.05$). However, testis weight was approximately one third higher ($p < 0.05$) in KO mice. The results already found showed that the volume density (%) of the seminiferous tubules and Leydig cells were, respectively, 93±0.4 and 3.7 ± 0.2 in control and 94.8 ± 0.2 and 2.2 ± 0.2 ($p < 0.05$) in iNOS KO. The data obtained for the Leydig cell nuclear volume was the same in both groups investigated. However, the cytoplasmatic and individual cell volume were lower ($p < 0.05$) and the total number of Leydig cells tended to be higher in iNOS KO mice. Based on the development of the acrosome, XII stages were also characterized for iNOS KO mice. We observed also significant differences in stages I (↑50%), VII (↓35%) and XI (↓50%) frequencies in iNOS KO mice. In this regard, we are currently investigating the seminiferous epithelium cycle length in these animals using tritiated thymidine. Regarding the cell counts, the numbers of Sertoli cells nucleoli, type A spermatogonia, and preleptotene spermatocytes per tubular cross-sections, evaluated at stage VII, were similar ($p > 0.05$) in the both groups investigated, whereas round spermatids presented lower population in these tubules. However, the total number of Sertoli cells per testis was almost 30% higher in iNOS KO mice, and the Sertoli cell efficiency (number of spermatids per Sertoli cell) were approximately 40% lower ($p < 0.05$) in iNOS KO. On the other hand, daily sperm production (spermatogenic efficiency) per testis in iNOS KO mice tended to be lower (~22%). Taken together, these preliminary data suggest that nitric oxide synthase might play an important role in spermatogenesis, particularly during spermiogenesis, and also in Leydig cells function and in the proliferation rate of Sertoli cells. However, more studies are still necessary (including a higher number of KO mice and probably hormonal assays), in order to get a better understanding about the mechanisms involved in the iNOS effects on the testis function.

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Duration of spermatogenesis in bullfrog (*Rana catesbeiana*)

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Introduction

Besides having economical importance, the bullfrog (*Rana catesbeiana*) has been utilized as an experimental model for biological studies such as pharmacology, medicine and reproductive biology, particularly those related to gametogenesis. Moreover, in order to investigate the phylogenetic plasticity of Nile tilapias as a recipient model for germ cells transplantation, ongoing studies in our laboratory is utilizing the *Rana catesbeiana* as a donor for germ cells. However, there is little information in the literature regarding the testis structure and function for this amphibian species. In this regard, we are currently developing accurate histological and stereological studies in bullfrogs testis. Particularly, we are interested in the estimation of the duration of spermatogenesis in this species, information that is crucial for tracking transplanted bullfrog germ cells into the testis of sexually mature tilapias.

Materials and Methods

Approximately thirty adult males bullfrogs received intracelomic injections of tritiated thymidine and had their testes analyzed between 1 hour and 35 days (2 days interval between sampling) after injection. After radioautography routine processing, analyses of the obtained testis sections were performed by light microscopy in order to detect the most advanced germ cell type labeled in each time period considered.

Results and Discussion

As shown in Figure 1, the duration of spermatogonial, spermatocytary and spermiogenic phases of spermatogenesis were 18, 14, and 8 days, respectively. It means that the total duration of the spermatogenic process was 40 days, a value similar to the majority of mammalian species investigated (1, 2) and much shorter than the results obtained for most fish species (3). Also, in comparison to other vertebrate species, the relative duration of spermiogenesis in bullfrogs is very short and, particularly, 4 days faster than the value cited for the other amphibian species (12 days, X*enopus laevis*) already investigated (4). In conclusion, to our knowledge, this is the first accurate report for the duration of spermatogenesis in any amphibian species.

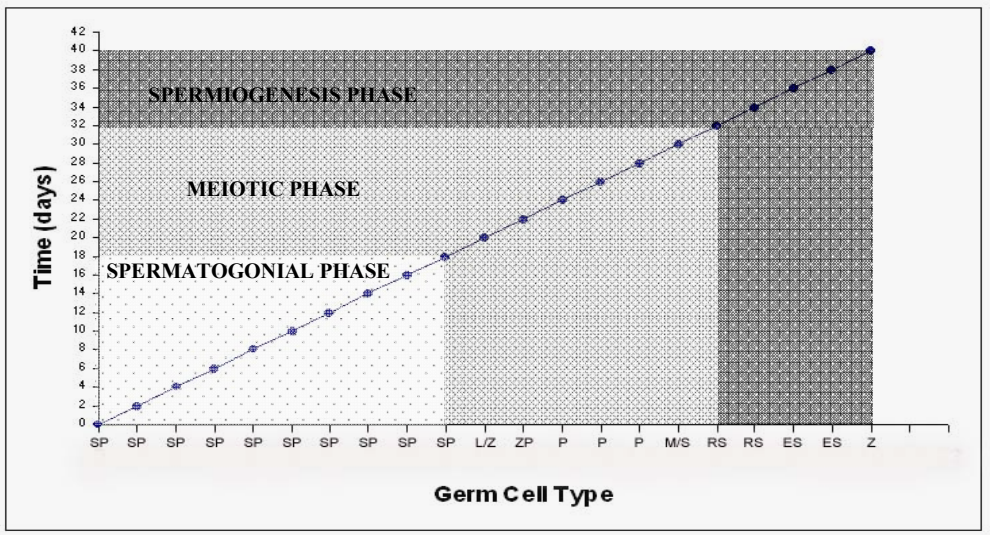

Figure 1. Duration of the three phases of spermatogenesis in *Rana catesbeiana*.

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Ultrasonographic study of testicular development in young Nelore bulls raised in extensive management system

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Introduction

Ultrasonographic evaluation of testes is a fast and non-invasive method which associated to clinical data can lead to a premature diagnosis of testicular disorders. Ultrasonographic studies of testicular development in young *Bos Taurus* bulls concluded that echogenicity of the testicular parenchyma presents a positive correlation with the animals age. In addition, pubescent animals present greater testicular echogenicity when compared to non-pubescent ones at the same age. The aim of the present study was to evaluate the ultrasonographic testicular image pattern, promote a quantitative analysis of ultrasonographic testes images, establish normal physiological patterns, and define the role of echogenicity as a sexual precocity marker.

Material and Methods

Testes of 19 young Nelore bulls were submitted to ultrasonographic evaluation with 8 MHz linear probe at 9, 11, 13, 15 and 18 months old. All animals were raised in extensive management system with mineralized salt supplementation. Frontal and transverse planes of left and right testes areas were selected, and transferred to the computer by Echo Image Viewer (EIV) software. Two interest areas (IA) of each scanned plan of left and right testicles were selected. Each interest area was bounded by a 6.3mm square, containing maximum testicular parenchyma (without embodying skin, tunics, epididymis, and testicular mediastinum). Testicular echogenicity of IAs were automatically determined by EIV software, in a zero (anechoic) to 100% (hyperechoic) gray scale. Tukey's test in SAS statistical software was used to compare means of each interest area among ages ($P < 0.05$). Semen samples were collected by electroejaculation when the animals completed 18 months old. Herein sperm count with $50x10^6$ spermatozoa, with at least 10% of progressive motility was established as puberty criteria.

Results and Discussion

Homogeneous and low echogenicity testicular parenchyma was observed in all animals. Testicular echogenicity increased in proportion to the animals age. Testicular parenchyma echogenicity at 9, 11, 13, 15 e 18 months were 18.5%, 28.5%, 25.2%, 29.5% e 45.2%, respectively. Only values obtained at 11 and 15 months did not present significant statistical difference ($P > 0.05$). Six animals presented semen quality compatible to puberty at 18 months-old, and similar testes echogenicity to non-pubescent animals at the same age. The increased echogenicity can be assigned to the entire spermatogenesis process that increases cellular density, as well as seminiferous tubules anatomic changes. The decrease of echogenicity can be explained by the formation of the seminiferous tubules lumen, such as fluid production by the Sertoli cells. In conclusion, ultrasonography is not a good indicator of sexual precocity once there was no significant difference ($P > 0.05$) between testicular echogenicity of pubescent and non-pubescent animals at the same age.

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The use of metallic implants to facilitate follicular dynamics study in ewes

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Introduction

The reproduction biotechnologies are important to increase the productivity in the domestic animal breeding, outstanding the estrus synchronization as an important technique. Ultrasound assessment of the female reproductive tract enables the evaluation of the ovary dynamics *in vivo*, which in the past was only accessed by invasive methods like ovariectomy or laparotomy/laparoscopy of animals in different days of the cycle(1). However, a fast and accurate examination is an important aspect to consider, especially considering small ruminant practice. Bearing this in mind, the present study aimed to evaluate the effectiveness of ovarian metallic implants to locate and facilitate the evaluation of the ovarian activity in ewes after a synchronization protocol using *d*-cloprostenol ($PGF_2\alpha$).

Material and Methods

Eight crossbreed ewes previously synchronized were housed at the ovine and goat reproduction applied biotechnology laboratory of FMVZ – UNESP, Botucatu, located at 22º53'09" South, 48º26'42" West and with 804 meters of altitude, during the month of May. The females were divided into two experimental groups: Control Group (without ovarian implant) and Implant Group (surgical steel implant placed at the proper ligament of the ovary; on the left ovary (LO) a stainless steel ball (Ø = 3mm) implant whereas a spiral metallic (Surgical Suture - PolySuture™, nº3) implant was placed on the right ovary (RO). To begin the experiment all ewes received an intramuscular injection of d-cloprostenol (150µg) (Day 0), starting then the ultrasound exams every 6 hours till the detection of ovulation. The equipment used was the Aloka™ SSD-500, connected to a prostatic linear probe of 7.5MHz. On Day 1 all ewes were teased every 6 hours, after the ultrasound evaluation, until the end of the oestrous behavior.

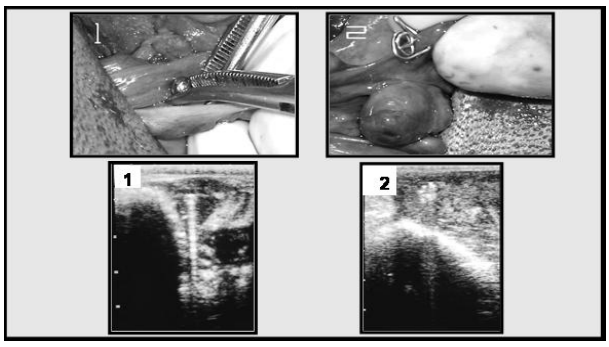

Figure 1. Placing the implant at the proper ligament of the left ovary (1) and right ovary (2), and their respective implant ultrasound images.

Results and Discussion

It was possible to clearly differentiate by ultrasound devices implanted (Fig. 1). At the third evaluation on Day 1 (D1C) the ultrasound examination was faster in the Control Group than in the Implant Group ($p < 0.05$). At the first evaluation on Day 3 (D3A) the ultrasound exam was faster in the Implant Group when compared to the Control Group ($p < 0.05$). At the other evaluations no difference in the examination time was found ($p > 0.05$) between groups. The estrus, ovulation intervals and the total duration of the estrus behavior did not differ between the studied groups, the average between the application of $PGF_2\alpha$ and the ovulation time of 70 and 74 hours for the experimental group and the control group, respectively, no difference was found ($p > 0.05$). In this study it was not found any advantage in the use of ovarian implants in ewes in order to reduce the time of the ovary ultrasound evaluation. The time to perform the examination seemed to be related to the animal behavior and had a great variation among moments. Further studies using more animals should be performed to reduce the female factor, to better access if there efficiency may improve using the ovarian implants. However, the implant showed that ovaries switched sides in 3 of 43 ultrasound exams, thus showing that implants were good evidence for ovary side control avoiding mistakes during the ovarian evaluation in ewes, and being of good use on ovarian ultrasound training programs.

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Morphological and functional features of corpora lutea induced by administration of cloprostenol in goats

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Introduction

A common way for synchronization of estrus and ovulation in goats, either for natural breeding or artificial insemination, is the use of two PGF2α doses, 10-12 days apart, during the breeding season (1). However, the yields obtained by the use of this protocol are still very variable; a possible cause may be linked to alterations in the functionality of preovulatory follicles, giving way to defective corpora lutea unable to maintain pregnancy (2). Thus, the objective of this study was to compare the patterns of luteal dynamics and function during the estrous cycle subsequent to natural spontaneous estrus or an estrus induced by the administration of a PGF2α analogue (cloprostenol) in goats.

Materials and Methods

Fourteen adult and cycling Anglo Nubian goats maintained at the experimental farm of the National University of Río Cuarto (Argentina, latitude 30º07'S) were used during the breeding season. Does were randomly allocated to two groups: Group N (n = 7) included goats with natural spontaneous estrus and Group PG (n = 7) included does in which estrus was synchronized by the administration of two i.m. cloprostenol doses, 10 days apart. In both groups, estrus behavior was checked twice daily with a vasectomized buck (Day of estrus = Day 0). The position and size of corpus luteum (CL) were recorded by daily transrectal ultrasonography for evaluating their development through the complete subsequent estrous cycle. The luteal activity was determined directly, in terms of progesterone (P4) secretion, by drawing jugular blood samples coincidentally with ultrasonographies, and indirectly, by assessing effects of CL on follicle dynamics through evaluation of the number, position and diameter of all ≥2mm follicles present in the ovaries.

Results and Discussion

All goats exhibited estrus behavior and ovulation, without differences in ovulation rate (N: 1.67 ± 0.2, PG: 2.0 ± 0.1). The total luteal tissue (CL) area showed a linear growing from Day 4 to Day 15 of estrous cycles in all the goats, but the growth dynamics differed between groups N and PG (Figure 1A); when analyzing correlations between day of cycle and CL area (N: r = 0.243; P < 0.001 and PG: r = 0.643; P < 0.001) mean values were higher in group PG (P < 0.01). Plasma P4 concentrations also increased from Day 0 to Day 15 in all does; however, from Day 5 to Day 15, group N had higher concentrations than group PG (P < 0.001; Fig. 1B). In each group, P4 concentrations were positively correlated with CL area (N: r = 0.645, P < 0.0001; PG: r = 0.857, P < 0.0001). Cycles with 3 waves were most comm''on pattern observed (N: 67%; PG: 60%). There were differences in follicle development; diameter of dominant follicles from the first and second waves were higher in group PG (7.6 ± 0.8 and 6.3 ± 1.4mm) than in group N (4.9 ± 0.7 and 5.0 ± 0.4mm; P < 0.05). In the ovulatory wave, the diameter of preovulatory follicles was 8.3 ± 0.4 and 7.2 ± 0.4 for PG and N, respectively (P < 0.05). These results suggest that the luteal function may be compromised in animals treated with PG and, thus, may influence the decrease in fertility rates classically reported.

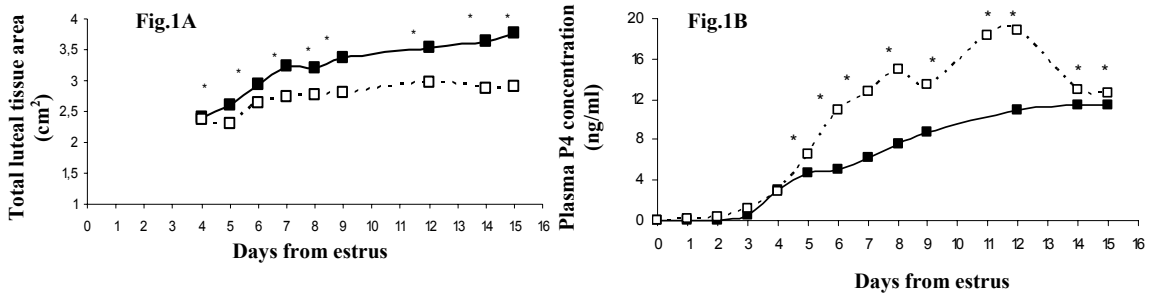

Figure 1. Total luteal tissue area (Fig. 1A) and plasma P4 concentrations (Fig. 1B) for N (white square) and PG (black square) groups from Day 0 to Day 16 of estrous cycle. Asterisk (*) indicates P < 0.001 between groups.

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Ultrasonographic pattern, Color and Flow Doppler of the testis and epididimus of Botos (Amazon River Dolphin) *Inia geoffrensis*

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Introduction

Riverine dolphins are among the least known all cetacean. Their great ecologic value has lead many researchers to make efforts in order to obtain more information about their habitat, behavior and reproductive characteristics (1). Ultrasonographic examination has been shown to be an excellent tool to present particularly valuable details in morphological description and to physiologically evaluate reproductive events, as a safety, and repeatable technique (2).

Material and Methods

Live botos were examined using ultrasound during once-yearly capture expeditions, between October or November (2005), within the Mamirauá Sustainable Development Reserve, Brazilian Amazon (3◦S, 65◦W). All examinations were performed with a *Sonosite 180 plus* ultrasound unit in conjunction with a 5-7.5 MHz multifrequency transducer convex array *180 Plus/Elite-C60*. The transducer was covered by acoustic gel which displaced any air and provided good acoustic contact. All cross-sectional measurements of the testis could be made directly from the image display, using the in-built electronic caliper functions. However, the maximum width of the ultrasonographic field of view available was 10cm and it was not possible to take longitudinal measurements of larger testis directly from the monitor image. During this period 15 examinations were carried out, involving adult male Amazon dolphin. Age assessments and estimates of maturity were made from body length and weight and external characteristics.

Results and Discussion

No significant ranges were showed on the ultrasonographic appearance of the testes from adult male Amazon Botos. This characteristic has been seen in sub-adult male of *Tursiops truncatus aduncas* and many others mammalian species (3). In all examination the testes were discerned by a hyperechoic central line, which constitute a landmark to identification of testes during ultrasonography examination. In adult male botos, the parenchymal nodulations and mid-third epididymides enlargement revealed their reproductive age (4). The blood flow through the artery and vein was detectable, running medially and dorsally among the testis and epididymis. Testis' mediastinum showed well identified blood flow, decreasing while approaching the periphery, with diffuse points into the parenchymal testis. Little blood flow could be identified by Color Doppler. However, Flow Doppler allowed better accuracy to discern testicular vessels, their topography and adjacent structures. Ultrasonographic examination provides useful data for testis morphological characterization of male Amazon Botos. Examination by Doppler ultrasound was considered as a valuable tool in order to make evident the blood flow through the testicular parenchyma.

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Follicular dynamics of *Bos indicus, Bos taurus* and *Bubalus bubalis* heifers treated with norgestomet ear implant associated or not to injectable progesterone

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Introduction

Several hormonal protocols have been developed to synchronize the onset of follicular wave and ovulation. In one of these studies, the use of injectable progesterone (P4) at the beginning of the protocol (time of intravaginal P4 device insertion) was tested in Nelore (1). The authors observed that the animals treated with P4 showed better synchronization of the follicular wave emergence than those not treated. However, no effect was observed on the ovulation at the end of the protocol. Therefore, our hypothesis is that the treatment with injectable P4 is efficient in synchronizing the follicular wave emergence in *Bos indicus, Bos taurus* and *Bubalus bubalis* heifers treated with norgestomet ear implant.

Materials and Methods

A total of 30 heifers (10 Nelore, 10 Holstein and 10 Buffaloes) contemporarily raised were assigned in two experimental groups. On a random day of estrous cycle (D0) the animals were treated with EB - 2mg of estradiol benzoate i.m. (EB; Gonadiol®, Intervet Schering-Plough, Brazil) or EB+P4 - 2mg of EB plus 50mg of injectable progesterone i.m. (Progesterona Inyectable Oleosa 20mg/ml®, Syntex, Argentina). On this same day, all animals received a norgestomet ear implant (Crestar®, Intervet Schering-Plough, Brazil). On Day 8, the implants were withdrawn and a $PGF_{2\alpha}$ dose i.m. (Preloban®, Intervet Schering-Plough, Brazil) was injected. Fourty-eight hours later, it was administered a dose of GnRH (Gestran Plus®, Tecnopec, Brazil). Except by the plasmatic levels of P4 on D0 (P4L), all following variables were evaluated by ultrasonographic examinations: diameter of the CL on D0 (DCL), diameter of dominant follicle on Day 0 (DF0), time of the follicular wave emergence (E), number of follicles at the wave emergence (#FE), diameter of dominant follicle on Day 8 (DF8), diameter of ovulatory follicle (DOF), ovulation rate (OR) and time of ovulation after implant removal (OVT). Data were analyzed by PROC GLIMMIX in SAS program.

Results and Discussion

No interactions were observed among genetical groups and treatment with P4. Also, there was no effect of genetical groups on variables, except by the higher level of plasmatic progesterone observed in buffaloes (P = 0.0079), and by the greater number of follicles recruited at the onset of follicular wave in Nelore (P = 0.0003). Effects of treatment are summarized in Table 1. In conclusion, there was no effect of P4 in synchronizing the follicular wave emergence and ovulation of Nelore, Holstein nor Buffaloes treated with norgestomet ear implant.

Table 1. Ovarian data from Nelore, Hosltein and Buffalo heifers treated with EB or EB+P4 at the beginning of hormonal protocol. Pirassununga – SP, 2008.

	NELORE		HOLSTEIN		BUFFALO		P Value
	EB (n=5)	EB+P4 (n=5)	EB (n=5)	EB+P4 (n=5)	EB (n=5)	EB+P4 (n=5)	
P4L (ng/ml)	2.3 ± 0.9	2.1 ± 1.1	2.9 ± 1.2	2.2 ± 1.0	4.9 ± 0.8	5.9 ± 1.0	0.99
DCL (mm)	16.4 ± 1.9	19.0 ± 4.3	18.3 ± 3.1	27.5 ± 4.8	19.1 ± 1.7	18.9 ± 1.5	0.13
DF0 (mm)	9.6 ± 1.3	10.0 ± 1.0	10.1 ± 1.8	14.4 ± 2.4	11.2 ± 0.9	10.5 ± 0.7	0.28
E (days)	4.6 ± 0.2	3.8 ± 0.2	4.4 ± 0.2	4.4 ± 0.2	4.4 ± 0.4	4.6 ± 0.2	0.37
#FE	30.0 ± 5.2	29.4 ± 4.2	12.8 ± 3.0	17.2 ± 4.8	13.0 ± 2.2	13.2 ± 1.9	0.67
DF8 (mm)	8.1 ± 1.3	9.3 ± 0.8	8.8 ± 0.6	8.4 ± 1.0	7.0 ± 0.8	8.9 ± 1.2	0.27
DOF (mm)	12.0 ± 1.8	12.4 ± 1.5	11.2 ± 0.6	11.0 ± 1.0	13.0 ± 2.0	12.2 ± 1.3	0.84
OR (%)	60.0	80.0	100.0	80.0	40.0	60.0	0.70
OVT (h)	78.0 ± 0.0	81.0 ± 3.0	78.0 ± 3.8	75.0 ± 3.0	84.0 ± 6.0	78.0 ± 6.9	0.57

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Preliminary study of early ultrasound diagnosis of pregnancy and fetal development in the Cutia *(Dasyprocta sp)*

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Introduction

Wild animal investigation has become more interesting in the scientific world due to its importance for ecology. Among a variety of species, rodents represent a great population in the world and a quarter is found in Brazil (1). Ultrasonographic examination has been shown to be an excellent tool to provide particularly valuable details in morphological description and to evaluate physiologic reproductive events, as a safety, and repeatable technique (2).

Materials and Methods

Females were clinically examined using vaginal swab and, with spermatozoa identification under light microscopy, we determined a positive mating, and gestational day zero. Twelve females were investigated using a PIE MEDICAL *Falco Vet* ultrasound and a convex multi-frequency transductor (5 and 7.5 MHz). Carbogel® was used as a contact medium for image enhancing. Animals were dorsally positioned; forelimbs and legs were gently unfastened and, an abdominal scanning was proceeded. No sedation or anesthesia was required during the procedure.

Results and Discussion

At 18th day of gestation, a well defined echogenic gestational sac composed by thin wall was observed; in its interior it was possible to identify a hypoechogenic embryo demonstrating a homogenous echotexture, as also related in human (2). In agreement to the descriptions in dogs, at 23rd gestational day, cutias' uterine wall demonstrated three well defined layers. Mother-fetal relation was verified with the presence of umbilical cord which was cleared identified due to the presence of amniotic liquid. Through the ultrasonography examination the sub-placenta was identified as a well defined discoid and heterogeneous echotexture, characterized to its dichotomized internal vascularization. This large structure was firstly described in pregnant rabbits during a morphological study (3). From the 40th gestational day, it was clearly identified a sub-placenta and fetus relation. At 55th day, a posterior acoustic shadow of vertebral column was identified, as well as, the hyperechogenic lung caudally to the cephalic region, in agreement to the descriptions in dogs (4). Acoustic shadow increased due to calcium deposit between the 60th and 70th gestational day. This phase was characterized by the complete definition of cephalic, thoracic (lung, vertebral body and ribs) regions and, liver presence. We concluded that the ultrasonographic exam in *Dasyprocta sp* was capable to identify from early stages of embryo development to fetal end-term, and development of internal organs, as well as the definition of embryonic membranes during gestational period.

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Pregnancy evaluation of ovine clone obtained by somatic cell nuclear transfer

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Introduction

Animal cloning by the somatic cell nuclear transfer technique (SCNT) is still inefficient. Less than 5% of the reconstructed embryos usually develop pregnancy at term, partially due inadequate placentation (1, 2). This paper aim was to evaluate the pregnancy of an ovine SCNT clone through fetal viability (fetal heart rate- FHR) and placentomes development by ultrasonography (US), and also progesterone (P_4) profile evaluation of the recipient.

Materials and Methods

Oocytes obtained from follicular aspiration by laparoscopy or by oviduct flushing were used as nuclear receptors of adult fibroblastic cells. Two to four embryos were transferred to 15 recipients. Pregnant recipients were monitored through plasmatic progesterone profile (P_4) by radioimmunoassay (DPC®, Los Angeles, USA) and by ultrasonographic examinations which started on the 25th day after estrus (5MHz linear transducer, Aloka SSD 500, Tokyo, Japan). Measurements concerning FHR, length and width of 4 to 5 placentomes close to the fetus were performed weekly until the 60th day and every 10 days till the end of gestation. Ovine fetuses and placentas from natural mating were used as control. In order to analyze data, pregnancy was divided in first trimester (1; until 51 days), medium trimester (2; from 51 to 100 days) and final trimester (3; more than 101 days). Results are expressed by mean and standard error and evaluated by analysis of variance (ANOVA) by GLM procedure, SAS, version 9.1.3 (SAS Inst. Inc., Cary, NC, USA); differences between means were analyzed by "T" test with significance level of 5%.

Results and Discussion

Only one pregnancy from a recipient inovulated with 2 embryos came full term. It was observed in this pregnancy great decrease in plasmatic concentration of P_4 (17.23 ng/mL vs 9.11ng/mL) between the 51st and 60th day of gestation, confirming the death of one of 2 embryos, evidenced by US. The FHR, evaluated only during the medium and final trimester, decreased (198.25 ± 6.6 to 159.4 ± 4.7 beats/min; $p < 0.001$) reaching 144 beats/min on the 148th day, which is the physiological moment when gestation ends in this species. Placentome length ($p = 0.003$) and width ($p = 0.032$) were greater for the clone placenta (length = 2.95 ± 0.11; width = 2.53 ± 0.10) than for control (length = 2.66±0.07; width = 2.14±0.1). It was found effect of pregnancy trimester on placentome length and width in both clone placenta (trimester 1 = 2.14 ± 0.16; 1.8 ± 0.15cm; trimester 2 = 3.37 ± 0.07; 2.7 ± 0.06cm; trimester 3 = 2.9±0.09; 2.5±0.08cm; $p < 0.001$) and control fetuses (trimester 1, length = 1.85 ± 0.15; width = 1.2±0.13cm; trimester 2, length = 2.98 ± 0.04; width = 2.1 ± 0.03cm; trimester 3, length = 2.41 ± 0.06; width = 1.8±0.05cm; $p < 0.001$). Placentome size/weight decrease at term (145-150) is described as a physiologic reaction and happens due to a structural rearrangement to increase the capacity of nutrient transfer when fetal demand increases (3). Fetal heart rate monitoring associated to its movement decrease during the ultrasonographic examination on the 148th day of gestation, evidenced fetal suffering. This demonstrates that ultrasonography is an important tool to control high risk pregnancies, as of small ruminant clones which due to their size allow access and monitoring by this technique.

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Day of follicular wave emergence of holstein heifers and cows submitted to protocols for synchronization of follicular wave emergence using or not injectable progesterone

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Introduction

The success of *in vitro* embryo production (IVP) is directly related to the number and quality *cumulus*-oocyte complexes that are assigned to culture. Some studies have shown the relevance of the follicular wave phase at the moment of *ovum pick-up* on oocyte quality and IVP. Thus, studies involving protocols to improve these aspects are extremely important. The aim of the present study was to determine the effect of the use of injectable progesterone (P4) in protocols for synchronization of follicular wave emergence in different categories of Holstein females: heifers, dry cows and high-producing lactating cows.

Material and Methods

The experiment was conducted on a commercial farm in southwest Brazil during August 2008. Thirty non-pregnant Holstein females of three different categories were used: 12 heifers, 6 dry cows and 12 high-producing lactating cows. The animals of each category were equally divided into one of two experimental groups: without P4 (G-NoP4) and with P4 (G-P4). Animals in G-NoP4 (n=14) received one Norgestomet ear implant (Crestar®, Intervet Schering-Plough, Brazil), 2 mg of Estradiol benzoate (Sincrodiol®, OuroFino, Brazil) and 500 mcg of Cloprostenol (Sincrocio®, OuroFino, Brazil), on a random day of the estrous cycle (D0). Animals in G-P4 (n=17) received same treatment except for the administration of one dose of 50 mg of progesterone 2.5% i.m. (OuroFino, Brazil) on the same day (D0). Five days later (D5), all animals had the implant removed. Ultrasonographic evaluations (Chison 600VET) were performed every 24 hours from D0 to D5 in order to determine the day of follicular wave emergence. Data were analyzed using the PROC GLIMMIX from SAS.

Results and Discussion

No effect among the three different categories of animal (heifer, dry and high-producing lactating cow) was detected. Also, no effect of P4 was observed on the day of follicular wave emergence.

Table 1. Effect of animal category on the day of follicular wave emergence and the number of follicles on D5.

	Lactating (n=12)	Dry (n=6)	Heifer (n=13)	P-value		
				Cat.	P4	Cat. x P4
Day of wave emergence	3.9 ± 0.2	3.5 ± 0.2	3.8 ± 0.2	0.22	0.13	0.20
Follicles ≥ 2 and < 6 mm	27.2 ± 3.93	37.0 ± 5.3	25.6 ± 3.9	0.45	0.14	0.40
Follicles ≥ 6 and < 10 mm	1.75 ± 0.51	0.8 ± 0.2	0.6 ± 0.3	0.13	0.75	0.99
Follicles > 10 mm	0.9 ± 0.26	0.8 ± 0.5	0.2 ± 0.1	0.13	0.85	0.51

Table 2. Effect of treatment with P4 on the day of follicular wave emergence and the number of follicles on D5.

	No P4 (n=14)	P4 (n=17)	P-value		
			Cat.	P4	Cat. x P4
Day of wave emergence	3.6 ± 0.2	3.9 ± 0.1	0.22	0.13	0.20
Follicles ≥ 2 and < 6 mm	24.7 ± 2.8	31.8 ± 3.9	0.45	0.14	0.40
Follicles ≥ 6 and < 10 mm	1.2 ± 0.4	1.00 ± 0.3	0.13	0.75	0.99
Follicles > 10 mm	0.7 ± 0.3	0.5 ± 0.2	0.13	0.85	0.51

The use of injectable progesterone associated to EB and ear implant did not seem to cause any effect regarding follicular wave emergence. Also, animal in all categories presented similar day of follicular wave emergence.

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Embryo production in superovulated Nelore heifers supplemented with rumen-protected fat

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Introduction

Supplemental lipid can be included in the diet of ruminants to increase energy density. Moreover, polyunsaturated fatty acids seem to exert an extra-caloric positive effect on ruminant reproduction, although the reasons for that are still unclear. While some studies have detected a positive effect of feeding unsaturated fatty acids on embryo development of superovulated *Bos taurus* cattle (1) others have not (2). The aim of the current study was to evaluate the superovulatory response and embryo production in Nelore (*Bos indicus*) heifers supplemented with rumen-protected fat.

Materials and Methods

Forty heifers kept in pasture (*Brachiaria decumbens*) were randomly divided into two experimental groups according to supplementation: F = supplemented with rumen-protected fat (600 g/heifer/d) and C = Control, without fat supplementation (900 g/heifer/d). The F diet was formulated to supply 100 g/heifer/d of a rumen-protected fat from soy oil, predominantly linoleic acid (Megalac-E, Arm & Hammer, Rio de Janeiro, Brazil). The supplements were formulated to be isocaloric and isoproteic. Each female underwent both treatments in a cross-over design with approximately 65 d between replicates. After 50 d of feeding the diets, the emergence of the wave was synchronized by an i.m. injection of 2.0 mg estradiol benzoate (Estrogin, Farmavet, São Paulo, Brazil) and insertion of an intravaginal progesterone releasing device (1.9 g progesterone, CIDR, Pfizer, Hamilton, New Zealand) on Day 0. On Day 4.5, the superstimulatory treatments (70 mg pFSH; Folltropin-V, Bioniche Animal Health; Belleville, Canada) were initiated and given in decreasing doses of 28, 21, 14 and 7 mg twice daily, over a 4-day period. At the time of the fifth and sixth injections of FSH, 25mg of dinoprost tromethamine (Lutalyse, Pfizer, Paulínia, Brazil) was injected i.m. The CIDR was removed at the time of the seventh superstimulatory injection. Ovulation was induced with an i.m. injection of 0.05 mg GnRH (Gestran Plus; ARSA S. R. L., Buenos Aires, Argentina) 12 h after the last superstimulatory injection. All heifers were artificially inseminated with frozen/thawed semen from the same bull 12 and 24 h after GnRH. Seven days after the first AI, embryos/ova were recovered using a nonsurgical uterine flushing technique and classified according to IETS standards. To determine the superstimulatory (number of follicles ≥6mm 12 h prior to GnRH) and superovulatory (number of ovulated follicles 48 h after GnRH, confirmed by CL number at the time of embryo collection) responses, transrectal ultrasonography was performed. Data were analyzed by paired t or chi-square tests and are presented as mean ± SEM.

Results and Discussion

There was no difference between F and C groups regarding superstimulatory (22.6 ± 2.0 *v.* 22.9 ± 1.6 follicles ≥6mm, $P = 0.88$) or superovulatory (15.6 ± 1.3 *v.* 17.7 ± 1.2 CL, $P = 0.25$) responses. Treatments resulted in similar number of total embryos/ova (9.0 ± 1.0 *v.* 10.1 ± 0.9, $P = 0.38$), viable embryos (3.9 ± 0.4 *v.* 4.7 ± 0.7, $P = 0.37$), degenerate embryos (3.8 ± 0.8 *v.* 3.5 ± 0.5, $P = 0.82$), or unfertilized oocytes (0.9 ± 0.4 *v.* 1.6 ± 0.5, $P = 0.28$) recovered. Contrary to the results reported by Thangavelu et al. (2007), in our study, viable embryos from the group supplemented with polyunsaturated fat seem to have presented a slower development in relation to the group not supplemented with fat (54 morulae [34.6%] and 102 blastocysts [65.4%] for the F group *v.* 41 morulae [22.0%] and 145 blastocysts [78.0%] for the C group; $P = 0.01$). To our knowledge, this was the first study that evaluated embryo production in superovulated zebu heifers supplemented with rumen-protected fat. Although no positive effect of supplemental fat was detected on embryo quality, the influence of polyunsaturated fat supplementation on embryo cryotolerance, or pregnancy rate deserves further investigation.

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Influence of number of ovarian follicles >5mm at time of embryo collection on embryo quality of superovulated cows

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Introduction

Number of follicles at time of embryo collection is highly variable in superovulated cattle. Some cows have follicles < 5 mm diameter while others have all classes of follicles, including large anovulatory follicles. It is known that growing follicles >5 mm secrete hormones as well as other stimulating or inhibiting factors of cellular growth. It as been speculated that embryo quality can be positively (1) or negatively (2) affected when exposed to high concentrations of these hormones (mainly estradiol). However, neither the exact timing of an estrogen effect nor the mechanism by which estrogen may interfere with the developing embryo have been determined. The aim of this study was to evaluate the relationship between the number of follicles >5 mm present on the ovaries at time of embryo collection and the embryo quality of superovulated cows.

Material and Methods

Fifty-six Mantiqueira dairy cows (a *Bos taurus* local breed) were superovulated with twice daily pFSH injections, during 4 days. On the morning of the third day the animals received a luteolytic dose of prostaglandin F2α analogue and mated with a bull at the induced estrous. Seven days after mating, the cows were slaughtered and the genital tract recovered for visual inspection of ovarian structures. The uterus was flushed with approximately 100 ml PBS (added of 10% bovine fetal serum), using a Foley catheter while the oviduct was injected (into the ampoule to uterus direction) with approximately 50 ml PBS, using a needle coupled to a syringe. The embryos were searched and morphologically evaluated (following IETS criteria) under a stereoscopy (x60 magnification). Data of embryo production and quality were submitted to linear regression analysis.

Results and Discussion

On average, 7.8 ± 0.6 (variation from 1 to 18) follicles >5mm were observed in both ovaries and 13.8 ± 0.9 structures (oocytes and embryos) recovered and from which 7.6 ± 0.6 were transferable embryos. Embryo quality was not related to the number of follicles >5mm at time of embryo collection ($R^2 = 0.002$; $F = 0.7$). These results suggest that factors secreted by follicles >5mm during or before the embryo collection does not seem to affect the embryo quality of superovulated cows.

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Swine oocytes: isolation, quantification and classification

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Introduction
Biotechnology has been developed to study and follow the steps of assisted reproduction, both animal and human. The swine, occupies a place of prominence because of their similar to human physiology, which makes it a potential donor of tissues and organs for xenotransplants, as well as for production of specifics proteins. At the same time, the production system of pigs's meat is becoming receptive to the implementation of new reproductive technologies. The objective of this study was to isolate oocytes from swine's ovaries obtained in slaughterhouse and later quantification and morphological classification.

Materials and Methods
Were used 10 cross breed (Landrace x Large White) slaughtered swine females with ages ranging from 5 to 6 months. The ovaries were transported in 0.9% saline solution. The follicular fluid (FF) was obtained per follicular puncture. The FF was transferred to a Petri dish containing saline solution. CCO were classified in grades, from 1 to 4 (1) according the characteristics of cumulus oophorus cells and the cytoplasm of the oocyte. The results were analyzed by the Statistical Analyses System (2). The data relating to the quantity of oocytes per animal were analyzed by ANOVA and, as was detected effect of animal, multiple comparisons were made by the test of Dunnett. The percentages of different grades of CCO were analyzed by logistic regression.

Results and Discussion
Morphologically the oocytes of swine are very similar to those of cattle; therefore the same classification system can be used. Animal effects ($P < 0.01$) were detected in the total number of oocytes isolated and in the number of oocytes of grades 2 and 4; however there was no effect for grades 1 and 3. It was also detected animal effects ($P < 0.01$) on the percentage of oocyte of grade 1. Probably, animal effects were related to development follicular phase. Studies indicate that morphological changes occur in the oocyte of the follicle dominant before the peak of LH and are concurrent with changes in follicular production of steroids, and these changes are regarded as prerequisites for the oocyte to acquire the ability for final maturation, fertilization and cleavage (3). The left ovary was more active and it was detected side ovary effect ($P < 0.01$) on amount of oocytes grades 2 and 4. Data from the literature show that the two ovaries do not works equally. In cows, sheep and goats 55 to 65% of ovulations occur in the right ovary, and in mare and sow 55 to 60% of ovulations occurs in the left ovary (4).

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Effect of thawing duration of in vivo produced ovine embryos on the pregnancy rate and embryo survival

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Introduction

Embryo handling and culture is one aspect of embryo transfer that must be constantly maintained in order to achieve optimum results (1). The objective of this study was to determine the effect of 1 to 20 min holding of frozen-thawed ovine embryos in commercial media prior to transfer on the pregnancy rate and embryo survival.

Material and Methods

Morulae or blastocyst stages, grade 1 and 2 were obtained 6 days after artificially inseminated Romanov and Charollais donors. Morphologically normal embryos were frozen with conventional freezing protocol (ethylene glycol) and stored in liquid nitrogen. Thawing was performed by first maintaining the straws in air for 5-6 sec then kept in 37°C water bath for 30 sec. Embryos were randomly assigned to be placed in holding media (ViGro Holding PLUS, AB Tech., Pullman, WA, USA) for 5 min (n = 42), 10 min (n = 38), 15 min (n = 22) or 20 min (n = 18) prior to transfer. Embryos were evaluated for stage of development after culture. After evaluation, intact embryos were transferred (two embryos per animal) into the recipient. Pregnancy rates were compared with chi-square test. Embryo survival rates were calculated as number of lambs born from double embryo transfer.

Results and Discussion

The pregnancy rate 55 days after transfer was not significantly affected by length of holding time, but embryo survive increased from 79% with a 1-5 min holding time to 100% with a 15-20 min holding time (Table 1). Morulae stages showed higher pregnancy rate regard to increasing length in holding media while a decrease was observed with blastocyst stages. Thus, post-thaw embryonic survival was impaired for the blastocyst but improved for morulae stages. The pregnancy and survival rates were higher when morulae stages were exposed to 10 - 20 minutes in the holding media than the blastocyst stage.

Table 1. Pregnancy and embryo survival rates of frozen-thawed ovine embryos exposed in holding media from 1 to 20 minutes before embryo transfer.

Duration of holding time (min)	n	Pregnancy rate (%)	Embryo survival (%)
1-5 (A)	42	64	79[a]
5-10 (B)	38	68	58[b]
10-15 (C)	22	58	71[a]
15-20 (D)	18	50	100[c]
Holding time * Stage of embryos			
A- Blastocyst	26	77[a]	75[a]
A-Morula	16	44[b]	88[a]
B-Blastocyst	22	71[a]	50[b]
B-Morula	16	60[a]	56[b]
C-Blastocyst	12	56[b]	79[a]
C-Morula	10	67[a]	75[a]
D-Blastocyst	8	25[c]	100[c]
D-Morula	10	100[d]	100[c]

Values within columns, by category, comparisons not followed by the same letter are significantly different ($P < 0.05$)

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Developmental competence of immature bovine oocytes using brilliant cresyl blue test

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Introduction

Immature oocytes are known to synthesize a variety of proteins, including glucose-6-phosphate dehydrogenase (G6PD) enzyme. This enzyme, a component of the pentose phosphate cycle, is active in the growing oocyte, but has much lower activity in oocytes that have completed growth (1, 2). The brilliant cresyl blue (BCB) is a vital blue dye that shows the intracellular activity of G6PD. The BCB stain test has been used to select competent heifer (3) and prepubertal goat (4) oocytes that are more suitable for *in vitro* maturation, fertilization and embryo development. Results from heifer oocytes stained by the BCB test showed a higher fertilization rate of BCB+ (blue cytoplasmic staining) than BCB- (colorless cytoplasm) oocytes (74.6, 45.1, and 61.8% for BCB+, BCB- and control, respectively). The objective of this study was to evaluate the BCB test as a useful criterion to select bovine oocytes with a higher developmental competence for *in vitro* embryo production systems.

Materials and Methods

Abattoir-derived ovaries were obtained and transported in 0.9% NaCl solution at 25°C to the Animal Reproduction Laboratory at Embrapa Temperate Climate in Pelotas-RS. Cumulus-oocyte complexes (COCs) were aspirated from 2- to 8-mm follicles and morphologically evaluated through a stereomicroscope prior to BCB exposure (26 μM) for 90 min at 39°C. Thereafter, oocytes were selected based on their cytoplasm coloration (BCB+ or BCB-). Then, COCs were washed three times in a modified PBS (mPBS) and randomly allocated into experimental groups as follow: a) control group (placed in culture immediately after their morphologically selection); b) mPBS group (exposed to mPBS for 90 min prior to culture); c) BCB- (showed a colorless cytoplasm prior to culture); and d) BCB+ (showed a blue cytoplasmic staining prior to culture). The COCs were cultured *in vitro* from 18 to 20 h, at 39°C in a 5% CO_2 humidified air atmosphere. After maturation, COCs were transferred to 400 μl of fertilization medium (IVF-TALP) and immediately inseminated with a final concentration of 1 x 10^6 spermatozoa/mL. After fertilization period (18-20 hs), presumptive zygotes were cultured with SOFaa medium. The development rate (% blastocyst/cleaved) was compared among treatments at Day 8 post-insemination. Fischer's exact test was performed to detect differences among treatment groups. The level of significance was set at $P<0.05$.

Results and Discussion

In the present study we showed that 45.6% of morphologically selected COCs were stained by the BCB test (BCB+). However, we found no difference between BCB-, BCB+ and control oocytes subjected to IVM (Table 1). Cleavage and blastocyst rates were significantly lower in COCs cultured in mPBS compared to other groups ($p < 0.05$). Cytoplasm of BCB+ oocytes turns blue because they did not reduce BCB to a colorless compound (Figure 1). Further studies with a larger number of bovine COCs are needed to determine if the BCB test is a useful method to select a more homogenous oocyte population to enhance *in vitro* procedures in bovine.

Table 1. Embryo development *in vitro* after selection of bovine oocytes using Brilliant Cresyl Blue (BCB).

	Oocytes IVM	Cleavage Rate (%)	Embryonic Development (%)	Global Rate (%)
Control	82	48 (58.5)	18 (37.5)	18 (21.9)
mPBS	63	14 (22.2)	4 (28.5)	4 (6.3)
BCB -	66	28 (42.4)	12 (42.8)	12 (18.1)
BCB +	92	42 (45.6)	15 (35.7)	15 (16.3)

* Data from 4 replicates were pooled.
Values in the same column with different letters (a vs. b) differ significantly (P<0.05).

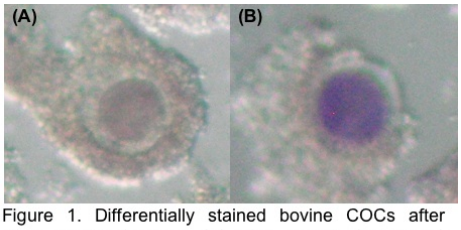

Figure 1. Differentially stained bovine COCs after exposure to BCB stain. (A) BCB- oocytes (unstained) and (B) BCB+ oocytes (blue stained).

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Search for auxin-responsive candidate genes in *Bos sp.* and *Ovis sp.* using a desk-top approach: potential tools for oocyte donor selection

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Introduction

Indole-3-acetic acid (IAA) belongs to an auxins group and presents benefic effects on animal metabolism, as related for improving sperm motility and providing longer period of semen preservation (1). It has been suggested that IAA binds to growth factors in the ovaries, playing an essential role to modulate these growth factors (2). Our team also described the *in vitro* activation of ovine primordial follicles after culture in media containing IAA (3). Despite these promising results, little information is available in the literature about the specific mechanism of how IAA works in animal cells. A complete knowledge of IAA action would be an important step to elucidate many processes involving the development of ovarian follicles. Our objective was to find out an association between possible roles of the IAA activity on preantral follicles with some candidate mammal genes, using a desk-top approach.

Materials and Methods

We selected genes related to stimulation of auxins in plants. After that, we retrieved their protein sequences from *Arabidopsis thaliana* genome at the GenBank database. Putative functions of each gene were obtained from the genome databank from *A. thaliana*, as well as those corresponding at the SWISS-PROT database. All amino acid sequences were used to perform a TBlastn in which the query amino-acid sequence was compared with genome sequences, expressed sequence tags (ESTs) or cDNAs translated in 6 frames. This search was done in default stringency criteria, against *Ovis sp.* and *Bos sp.* EST database to identify probable orthologous genes. The function of animal selected genes was verified at the SWISS-PROT and the metabolic pathways enrolled, using the Kyoto Encyclopedia of Genes and Genomes - KEGG. Selected sequences from *Arabidopsis thaliana, Bos taurus* and *Ovis aries* were aligned using the ClustalW program.

Results and Discussion

The gene search returned ten plant genes that seem to be extremely IAA-responsive. From those, the gene ROOTY (*Arabidopsis thaliana*) presented the best homologies score at *Ovis aries* (Identities: 40%, E-value $2e^{-39}$) and *Bos taurus* (Identities: 37%, E-value $1e^{-57}$) tyrosine aminotransferase (EC: 2.6.1.5) gene. The plant gene ROOTY looks to play an important role at the IAA metabolism and their correspondent mRNA were found when plant tissues were stimulated by IAA. Expression levels of the ROOTY orthologous gene (tyrosine aminotransferase) were described in: germinal vesicle-stage oocytes; in vitro derived embryos (2-cell, morula, blastocyst and nuclear transfer blastocyst); in vivo blastocysts and fetuses; corpora lutea; ovarian follicles; oviduct; endometrium and placenta/embryo from bovine fetuses, by Prather, R.S. (2002 unpublished data - GenBank Acess. # DN823420) from the *DNA Core Facility (Bovine Project)*. The complete role of the ROOTY for controlling auxin homeostasis in plants steel needs to be elucidated. However, the presence of its orthologus transcript in bovine oocytes and embryos suggests participation at the mammalian reproductive process. We conclude that ROOTY is an important candidate gene for playing cellular mechanisms on oocytes stimulated by IAA.

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Meiosis resumption in bovine oocytes induced by angiotensin II is mediated through AT_2 receptors

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Introduction

Angiotensin II (AngII) has been related with ovulation (1) and meiosis resumption in cows (2). However, the AngII receptor-subtype involved on meiosis resumption in bovine oocytes is unknown. The objective of this study was to investigate the AngII receptor-subtype (AT_1 or AT_2) involved in the blockade of inhibitory effect of the follicular cells on meiosis resumption of bovine oocytes and the role of bradykinin (BK) in this process. The hypothesis is that the AngII activates the AT_2 receptors in the follicular cells and stimulates the release of BK which acts as a mediator in the action of AngII on meiosis resumption of bovine oocytes.

Materials and Methods

The first experiment was designed to verify which receptor (AT_1 or AT_2) mediates AngII effect on nuclear maturation. Cumulus-oocyte complexes (COCs) were cultured for 7 or 12h in the presence of follicular hemisections and AngII (10^{-11}M), with or without one of the following AngII receptor-antagonists: losartan (AT_1-AngII receptor-antagonist; 10^{-6}M), PD123319 (AT_2-AngII receptor-antagonist; 10^{-6}M) or saralasin (non-selective receptor-antagonist; 10^{-7}M). A second experiment was designed to verify the role of BK in the action of AngII through AT_2 receptors. The COCs were cultured for 7 h in the presence of follicular hemisections and AngII (10^{-11}M), with different concentrations of HOE-140 (BK type B_2 receptor-antagonist; 0, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M). After culture, oocytes of all groups were denuded, fixed in a glacial acetic acid:methanol (1:3) solution and stained with 1% lacmoid. Oocytes were analyzed under an optic microscope (400x) and the rates of germinal vesicle breakdown (GVBD; after 7h of culture) or metaphase I (MI; after 12h of culture) were evaluated.

Results and Discussion

In the first experiment, the meiosis resumption in bovine oocytes induced by AngII was inhibit after competitive inhibition of AT_2 receptor with PD123319 (AT_2-AngII receptor-antagonist) or saralasin (non-selective receptor-antagonist). However, the selective inhibition of AT_1 receptor with losartan did not impaired the meiosis resumption induced by AngII (Fig. 1A). Similar response to the AngII receptor-antagonists was observed after 7 or 12h of culture. In the second experiment, there was no difference in the GVBD rate among groups treated with HOE-140 and AngII ($P > 0.05$; Fig. 1B). In conclusion, the positive effect of AngII on bovine oocyte nuclear maturation is mediated through the AT_2 receptors, independently of the BK/receptor B_2 pathway.

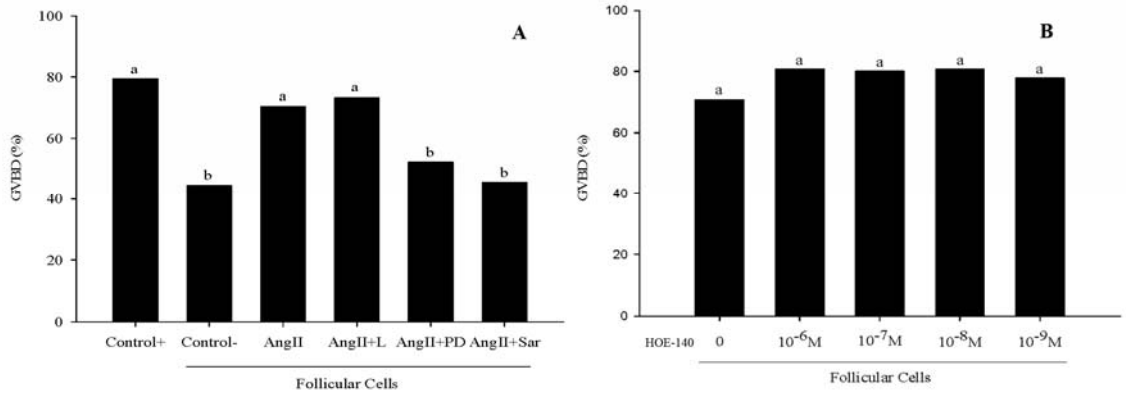

Figure 1. A) Effect of the losartan (L), PD123319 (PD) or saralasin (Sar) on meiosis resumption induced by AngII. B) Effect of different concentrations of HOE-140 on meiosis resumption induced by AngII. Both experiments were cultured for 7 h in the presence of follicular hemisections and significant differences are indicated by different letters ($P < 0.05$).

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Angiotensin II-induced meiotic resumption of bovine oocytes is inhibited by FGF10

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Introduction

Angiotensin II (AngII) is essential for meiotic resumption of bovine oocytes (1) acting through angiotensin receptor type 2 (AT2; Benetti et al., submitted), reversing the inhibitory effect of follicular cells on nuclear maturation on the co-culture system. Fibroblast growth factor 10 (FGF10) is expressed by oocytes and theca cells from bovine antral follicles and inhibits estradiol production (2) and AT2 expression in cultured bovine granulosa cells (3). The aim of this study was to test the hypothesis that FGF10 is an inhibitor of AngII-induced meiotic resumption of bovine oocytes, acting through follicular somatic cells.

Materials and Methods

Aiming to find the optimal dose of FGF10, cumulus-oocyte complexes (COCs; n= 320) were co-cultured with follicular hemisections (eight hemisections from 2 to 5 mm follicles in a 200 µL drop of maturation medium) in medium containing AngII (10^{-11}M) with FGF10 at 0, 10, 100 or 1,000 ng/mL. In a second experiment, COCs were cultured with or without follicular hemisections to test if FGF10 (100 ng/mL) acts directly on COCs or through follicular cells to inhibit AngII-induced meiotic resumption. In both experiments, grade 1 and 2 COCs were transferred into 200 µl of maturation medium with treatments specified above, and cultured for 7 h at 39ºC in a saturated humidified atmosphere containing 5% CO_2 and 95% air. At the end of the maturation period, oocytes were denuded, fixed in a glacial acetic acid:methanol (1:3) solution for 4 h and then stained with 1% lacmoid. The nuclear maturation stage was analyzed under an optical microscope (400x) and classified as germinal vesicle (GV) or germinal vesicle breakdown (GVBD).

Results and Discussion

The concentration of FGF10 that inhibited the resumption of meiosis induced by AngII presented a cubic trend ($P \leq 0.05$), indicating minimum germinal vesicle breakdown (GVBD) rate with FGF10 at 1,000 ng/mL (Fig. 1A). We used the concentration of 100 ng/mL for *in vitro* experiments based on previous reports showing the absence of detrimental effects on cell function (2). The AngII-induced GVBD (62.6%) was inhibited ($P \leq 0.05$) when FGF10 was added to *in vitro* co-culture system (37.8%; Fig. 1B). However, FGF10 did not affect ($P \geq 0.05$) meiotic resumption of COCs cultured without AngII in the presence or absence of follicular hemisections. The results showed that FGF10 does not alter the capacity of follicular cells to inhibit GVBD in absence of AngII and does not have a direct effect on COCs in spite of presence of FGF10 receptors in cumulus cells (4). In conclusion, the results demonstrate that FGF10 has an inhibitory effect on AngII-induced meiotic resumption, acting through follicular cells.

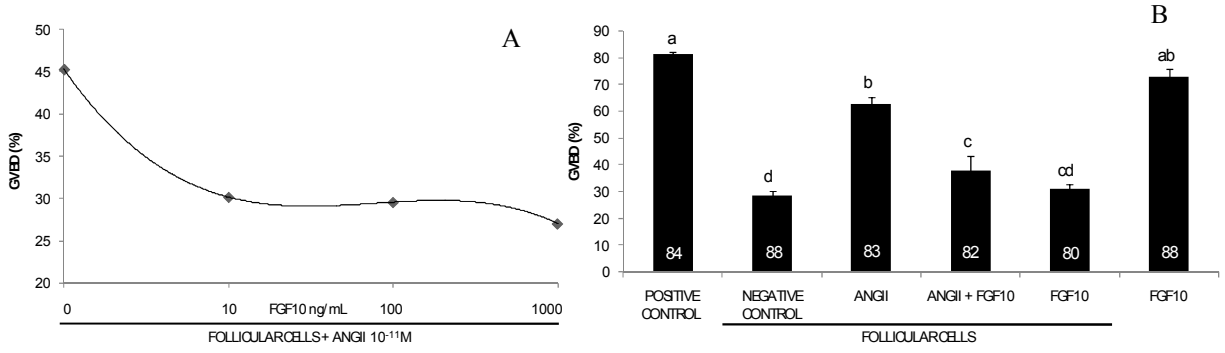

Figure 1. Effect of different concentrations of FGF10 on AngII-induced meiotic resumption (A). Meiosis resumption after 7 h of culture of bovine oocytes with or without follicular hemisections treated with FGF10 and/or AngII. Different letters indicate statistical significance ($P \leq 0.05$) between groups. The number of oocytes examined in each treatment is shown at the base of each bar (B).

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Re-utilization of norgestomet implants in superovulated Nelore cows

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Introduction:
The re-utilization of progestin implants is an alternative to reduce costs in protocols to induce ovulation in cows. The objective of this study was to evaluate the re-utilization of norgestomet implants in Nelore cows superovulated with eCG. The methodology proposed aimed the reduction of costs with superovulation protocols in Nelore embryo transfer programs.

Materials and Methods:
The experiment was performed at Embrapa experimental farm, located in São Carlos in the southeast of Brazil. Eight Nelore cows (*Bos taurus indicus*) were randomly distributed in two experimental groups. Group 1: On D0, a random day of the estrus cycle, half of the cows received a new norgestomet implant (Crestar®, Intervet) and 2 mg IM of estradiol benzoate (BE) (Estrogin®, Farmavet) and Group 2: On D0 the other half received 2 norgestomet implants, which were used before during 8 days, and also 2 mg of BE. On D4 cows from both groups received one single dose of 2000 IU of eCG (Folligon®, Intervet). On D6 two doses of $PGF_{2\alpha}$ (150 µg) (Sincrocio®, Ourofino) were administrated. The implants were removed 36 hours after the first $PGF_{2\alpha}$ and 12.5 mg of LH IM (Lutropin-V®, Bioniche) was administered 48 hours after the $PGF_{2\alpha}$. The cows were artificially inseminated 12 to 24 hours after LH injection with semen from just one bull. The number of follicles with ability to ovulate was estimated through transrectal ultrasonography (MINDRAY DP 3300) at the moment of LH administration (D8) and the ovulation rate was estimated on D15. The cows were flushed on D15 and blood samples were collected for progesterone radioimunoassay using LDH from FMVZ-USP. The results were analyzed through unpaired t Test and Fisher's Exact Test with a significant level of 5% ($P < 0.05$).

Results and Discussion:
The superovulatory response was higher on Group1. The mean number of corpus luteum (CL) and ovulation rate was also higher on Group 1. The results indicate that the superovulatory response with the re-utilization of 2 norgestomet implants in Nelore Cows is less intense when compared with the use of just one new implant.

Table 1. Results (Mean ± sd, when applicable) of superovulation in Nelore cows using new or used norgestomet implants:

	Group 1 (1 new implant)	Group 2 (2 used implants)
N° follicles >8mm	17.1 ± 1.8[a]	9.7 ± 1.6[b]
Follicular diameter (mm)	10.5 ± 1.6	10.2 ± 1.7
N° CL	13.8 ± 1.7[a]	5.5 ± 1.0[b]
CL diameter (mm)	15.7 ± 1.8	14.4 ± 1.7
Ovulation rate (%)	80.3[a]	53.8[b]
N° recovered structures	6.3 ± 1.1	4.0 ± 1.9
N° transferable embryos	4.0 ± 1.8	2.5 ± 1.1
[Progesterone] ng/ml[*]	36.1 ± 8.3	35.1 ± 6.9

[a,b]Different letters on the same line indicate statistical differences ($P < 0.05$).
[*]Progesterone measurements with 91% sensibility. The coefficient of variation intra assay was 9.1 and 5.6% and between assays 2.4 and 0.6%.

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A new approach to oocyte development in Ostariophysi

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Introduction

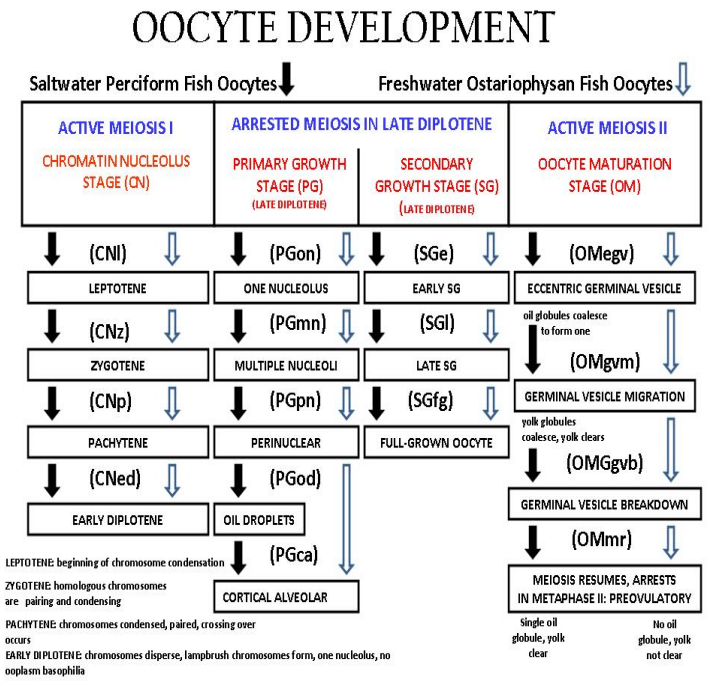
Recent reports on the role of the germinal epithelium in ovarian follicle formation and on the functional events that drive oocyte development have provided a new understanding of folliculogenesis and oogenesis in fish (1,2,3). In a call for updating and standardizing the concepts and terminology utilized in studies on folliculogenesis and on oogenesis in fish, Grier et al. (3) are proposing a unifying terminology to be applied to fish oocyte development that may also apply to other vertebrates. However, most of the knowledge in this field comes from higher Teleostei, primarily a few marine Perciformes with an exception being one freshwater salmoniform, the Rainbow Trout (4). The cellular and structural aspects of folliculogenesis and most of oogenesis appear to be constants throughout oocyte development. However, studies regarding the freshwater Ostariophysi have shown some significant differences from other studied fish. The Ostariophysi is an assemblage of basal Teleostei comprising the Cypriniformes, Siluriformes, Characiformes, and Gymnotiformes. Herein, we review follicle formation and oocyte development in some representatives of the Ostariophysi in order to make comparisons to marine perciform oocyte development.

Material and Methods

Females of the Siluriformes, *Pimelodus maculatus* and *Sorubim lima,* the Characiformes, *Serrasalmus maculatus* and *Schizodon nasutus*, and of the Gymnotiform, *Gymnotus sp*. were collected during the annual reproductive cycle in the Paraná-Paraguay basin. Their ovaries were fixed in Karnovsky's solution. For light microscopy, the material was embedded in historesin and sections were submitted to different staining methods. For transmission electron microscopy the material was post-fixed in 1% osmium tetroxide, block-stained in an aqueous solution of 5% uranyl acetate, dehydrated, embedded in Araldite, sectioned, and post-stained with a saturated solution of uranyl acetate in 50% ethanol and 0.2% lead citrate in NaOH (1N). Oocyte development was compared to the saltwater perciform, the Red Drum, *Sciaenops ocellatus*.

Results and Discussion

A comparative analysis of oocyte development among the species of Ostariophysi studied herein, and the data that is available regarding folliculogenesis in higher Teleostei, (1,2,3) shows that follicle formation from the germinal epithelium that borders the ovigerous lamellae must be constant throughout the Teleostei. However, oocyte development in the Ostariophysi differs from the Perciformes in some aspects. During primary growth, in saltwater perciform fish, oil droplets are deposited around the nucleus of the oocyte. The oil droplets become globules and coalesce into a single globule during oocyte maturation. Oil droplets do not occur in the ostariophysan fish examined herein (2). During oocyte maturation in the saltwater perciform fish, yolk globules fuse as hydration occurs. In Ostariophysi species, the yolk globules do not fuse and hydration does not occur. The yolk globules remain individualized even in the ovulated oocyte. The schedule presented here compares oocyte development in a saltwater perciform and freshwater ostariophysan fishes, showing the differences between them.



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Progesterone-induced bovine oocyte nuclear maturation is mediated by the COX pathway

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Introduction

The ovulatory LH surge promotes the meiotic resumption of bovine oocytes. However, bovine cumulus–oocyte complexes (COCs) do not have LH receptors and the LH effect must occur through follicular cells (1). Progesterone (P_4) and prostaglandins are two important players in the ovulatory cascade and they may be involved on the meiotic resumption of bovine oocytes. In vitro studies support the role of P_4 action in bovine follicular cells through its own receptor in the induction of mRNA for cyclooxygenase (COX) and prostaglandins secretion (2). However, the relationship between P_4 and COX in the meiotic resumption of bovine oocytes is unclear. Therefore, the aims of this study were to verify the potential role of P_4 in the meiotic resumption of bovine oocytes and to test the hypothesis that the COX pathway mediates this process.

Materials and Methods

The first experiment was designed to assess the P_4 effect on nuclear maturation of bovine oocytes cultured with follicular hemisections treated with 0, 10, 100, 1.000 or 10.000 ng/mL of P_4. After 22 h of culture, oocytes were denuded, fixed in a glacial acetic acid:methanol (1:3) solution and stained with 1% lacmoid. Oocytes were analyzed under an optic microscope (400x) and the metaphase II (MII) rate was evaluated. In a second experiment we verified whether P_4 action on meiotic resumption is mediated by COX. The COCs were cultured with follicular hemisections treated with 100 ng/mL of P_4 or 100 ng/mL of P_4 with 10 µM of indomethacin (a COX nonselective inhibitor). The COCs in the control groups were cultured with (negative control group) or without follicular hemisections (positive control group). After 7 h of culture, oocytes were denuded, fixed and stained as mentioned above. Oocytes were analyzed under an optic microscope (400x) and the germinal vesicle breakdown (GVBD) rate was evaluated.

Results and Discussion

In the first experiment we demonstrated that P_4 induces the nuclear maturation in bovine oocytes cultured with follicular cells in a dose dependent manner (Fig. 1A). The higher MII rate was observed when bovine oocytes were cultured for 22 h with follicular cells treated with 100 ng/mL of P_4 ($P < 0.01$). In the second experiment, the effect of P_4 on meiosis resumption was inhibited when 10 µM of indomethacin were added to the maturation medium (Fig. 1B). These results provide strong evidences that P_4 is involved on the meiosis resumption of bovine oocytes and that this event is mediated by prostaglandins pathway. However, further studies are necessary to elucidate how P_4 and COX interact on follicular cells function to promote meiotic resumption.

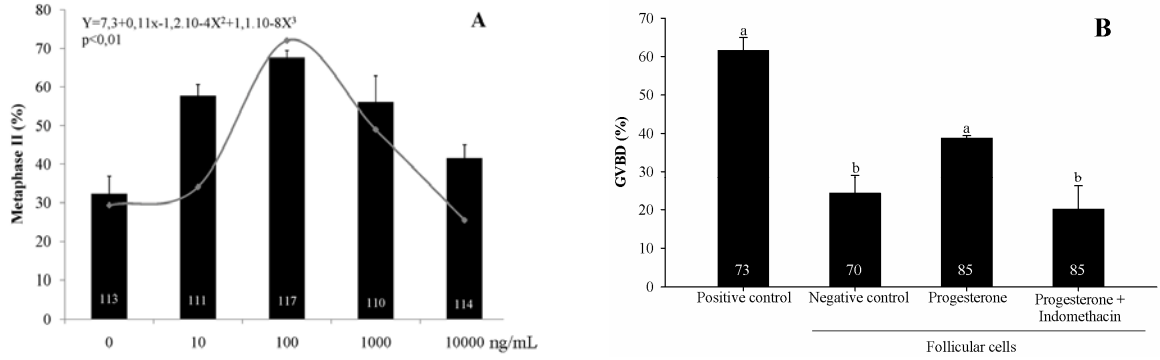

Figure 1. A) Metaphase II rate (solid bars) and predicted regression line after co-culture of bovine oocytes and follicular hemisections treated with different concentrations of P_4 by 22 h ($P < 0.01$). B) Meiotic resumption after co-culture of bovine oocytes and follicular hemisections treated with indomethacin and/or P_4 for 7 h. Significant differences ($P < 0.01$) are indicated by different letters.

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Development of the liver in Buffalo embryos during early pregnancy

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Introduction

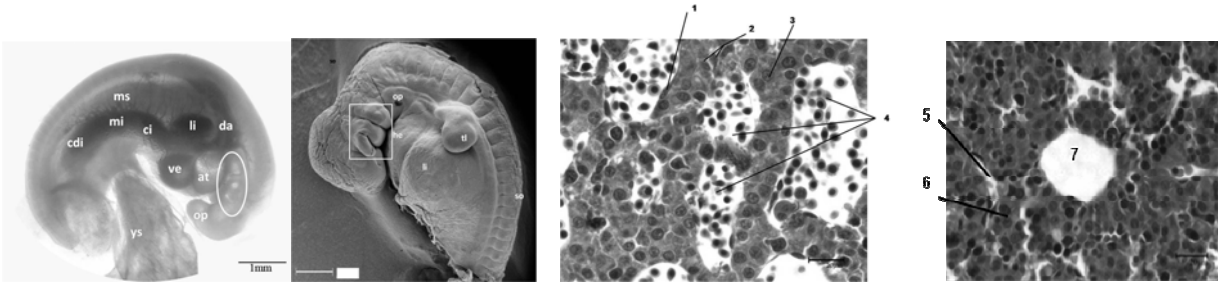
There are at least 130 million domesticated water buffalo (*Bubalus bubali*) in the World, which are classified as Swamp or River buffaloes. The Buffalo herd in Brazil, originally imported, currently amounts to around 400,000 head. The herd size is still increasing, especially in the lower Amazon region. Buffalo meat and milk are trades widely in Amazon towns and villages (1). Placentation and embryonic organogenesis of the water Buffalo was described previously (2, 3, 4), except for the early stages of implantation. The liver of bovine embryos at 4 weeks of gestations is proeminent on the embryo body (5). In humans, this organ has an essential role in hematopoiesis between the 2th and 7th months of the pregnancy (6). However, there are limited data on liver development in the bovine and none in Buffaloes. Thus, the present study aims to describe morphologic aspects of the development of the Buffalo liver between days 10 and 50 of gestation.

Materials and Methods

Buffalo embryos were collected from slaughterhouses locate in Macapá, in the Amazon region of Brazil and gestational stage estimated by Crow Rump legth (CR; 7). Next, whole embryos were fixed in Bouin solution, 3% paraformaldehyde or 2.5% glutaraldehyde for analyses by light microscopy, immunohistochemistry and scanning electron microscopy, respectively.

Results, Discussion and Conclusion remarks

The liver was the largest structure in the embryo body, occupying most of the abdominal cavity (Fig1B). Hepatocyte cords and small sinusoids with small blood cells inside were already observable in embryos with 20 days of gestation or less (Fig 1C). This was described also in humans (6, 8) and bovine embryos with 20, 29 and 34 days of gestation (9). The duodenum was caudal to the stomach, integrating the umbilical cord connected to the yolk sac (Fig 1A) and this was similar to bovines (8). The presence of sub-endothelial spaces, the space of Disse (8) was not found here, but others structures such as central vein and portal vein were observed. In embryos at 36 days of gestation, there was a clear development of the liver, as there was organization of the hepatoblasts, there was replacement of mesenchymal tissue and the presence of a large number of vessels and small capillaries sinusoids and other specialized liver cells (Fig 1D). Moreover, large vessels such as central and portal veins were also observed in this phase of gestation. Central veins may result from the the union of several capillaries present previously. There was positive immunostaining for PCNA in the liver cells, demonstrating the high proliferative activity between 16-20 days of gestation. There was no positive staining for Oct4 between 26-30 days of gestation, indicating the loss of pluripotency of cells during growth of the embryo. It was concluded that Buffalo liver morphology is similar to the bovine and there was evidence for high proliferative activity. The present findings can be used to verify the normality of embryonic development by ultrasound and other image techniques.



1A 1B 1C 1D

Figure 1. (A) Embryo with 0.7cm. Circle: brachial arcs; da, dorsal aorta; ve, ventricle; at, atrium; li, liver; ci, cervical intestine; cdi, caudal intestine; mi, medium intestine; ms, mesonephron, ys, yolk sac (B) Embryo with 1.0cm. square: branchial archs, tl, toracic limb; he, heart; li, liver; op, optic placode; so, somites. (C) Liver of the embryo with 1.1cm gestational age estimated in 24 days. HE stain 1.Endothelial cell 2.Kupffer cells 3. Hepatoblasts 4. Hepatic Sinusoids. (D) Embryo with 2.1cm estimated age of 36 days. 5. Sinusoid capilar 6. Hepatoblasts 7. Hepatic central vein. HE stain

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Evaluating the site of embryo deposit on pregnancy rates after bovine embryo transfer during summer and winter

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Introduction

Embryo transfer is a technique widely diffused all over the world in which more than 260.000 embryos annually are transferred in Brazil (1). However, not much is known about the ideal site to place the embryo into the uterine horn ipsilateral to the corpus luteum (CL; 2). Therefore, this study was designed to investigate the effect of the site of embryo placement (cranial, medial or caudal) into the uterine horn adjacent to the CL on pregnancy rates after fresh and frozen-thawed embryo transfer during two seasons of the year: summer and winter.

Materials and Methods

The experiment was conducted on a commercial farm in southeast Brazil during the winter of 2007 and the summer of 2008. Six hundred repeat-breeder (≥4 services) Holstein cows were used as recipients. They had their estrus either detected or induced by prostaglandin administration or synchronized for fixed-time embryo transfer. Embryos were produced *in vivo* using the technique of multiple ovulation and fixed-time artificial insemination and high-producing Holstein cows were used as donors. On embryo transfer day, recipients were randomly assigned to receive a fresh or a frozen-thawed embryo, which was deposited into the cranial, medial or caudal portion of uterine horn ipsilateral to the CL. A non-surgical embryo transfer was performed with morulae or blastocysts quality I, II and III (3). A technician with a working knowledge of embryo transfer carried out all transfers. Pregnancy diagnosis was performed 45 days after embryo transfer by transrectal palpation. Pregnancy rates were analyzed using Chi-square Test by SAS.

Results and Discussion

In summertime, the site of embryo placement did not influence pregnancy rates in recipients receiving fresh embryos ($P > 0.05$) [34.38% (22/64), 30.65 % (19/62) and 29.03% (18/62) for cranial, medial and caudal sites, respectively]. Also, no difference ($P > 0, 05$) was found on pregnancy rate in recipients receiving frozen-thawed embryos during this same season [34.72% (25/72), 33.8% (24/71) and 22.67% (17/75), respectively for sites previously mentioned]. Nevertheless, in wintertime, pregnancy rate was greater ($P < 00.5$) when fresh embryos were transferred to the caudal site comparing to the cranial one. However, no difference ($P > 0.05$) was detected when frozen-thawed embryos were placed into one of the three sites, in wintertime (Table 1). Despite these results demonstrate that the caudal site of the uterine horn adjacent to the CL may be the optimal site to place fresh embryos in winter, embryos can be deposited in any site of uterine horn ipsilateral to the CL in summertime. On the other hand, pregnancy rates related to frozen-thawed embryos were not affected by the site of embryo deposit in either season.

Table 1. Pregnancy rate after fresh or frozen-thawed embryo transfer into cranial, medial or caudal sites of uterine horn ipsilateral to the CL, during wintertime.

Uterine Site	% Pregnant (number pregnant/number of recipients)	
	Fresh embryos	Frozen-thawed embryos
Cranial	13.89 (5/36)[a]	17.86 (5/28)
Medial	28.95 (11/38)[ab]	13.79 (4/29)
Caudal	40.54 (15/37)[b]	32.00 (8/25)

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Development of *in vitro* produced bovine embryos in media with decreasing concentration of fetal calf serum

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Introduction

The majority of media used to culture bovine embryos *in vitro* contain fetal calf serum (FCS) or bovine serum albumin (BSA), and the benefit of these compounds on embryo development was long ago proved. However, serum appears to have a biphasic action, inhibiting the first's cleavages and stimulating embryonic growth after the morula formation (1). Also, the use of serum is correlated with large calf syndrome, metabolic and gene expression alterations and with an increase in the lipid content of *in vitro* produced (IVP) embryos (2). The increase in the lipid content has been associated with low embryonic quality and decrease in the successes after cryopreservation (3). This experiment aimed to compare the addition of different concentrations of FCS on bovine IVP media.

Material and Methods

After selection Grade I and II oocytes were *in vitro* matured in TCM-199 with Earle's salt + 10% FCS, FSH, LH and estradiol, during 22-24 hours in 5% CO_2 atmosphere. Semen was selected through Percoll gradient and the concentration adjusted to 1 x 10^6 sperm/mL. *In vitro* fertilization was performed in HTF media during 18-20 hours (D0). The presumably zygotes were culture in 90μL droplets of SOFaa + 0.6% BSA under mineral oil in a 5% CO_2, 5% O_2, 90% N_2 atmosphere, during 7 days, according with the following treatments: **Group 1 (control):** 2.5% FCS during 7 days; **Group 2:** 0% FCS during 7 days; **Group 3:** 0% FCS on days 1 to 3 and 2.5% FCS after day 3; **Group 4:** 0% FCS on days 1 to 3 and 5% after day 3; **Group 5:** 0% FCS on days 1 to 3 and 10% after day 3. Feedings were performed on days 3 and 5. Cleavage was accessed on day 3 and blastocyst formation on day 7. The results are based on 3 replicates. Data was analyzed through Chi-square at a significance level of 5%.

Results and Discussion

The results are summarized on Table 1. Based on the obtained results, no statistic differences were observed between groups concerning cleavage and blastocyst formation rates. These data indicate that, under conditions used in this experiment, the use of FCS is not necessary in any moment of embryo development. However a larger number of replicates are needed to confirm these results. Also the lipid content and survival to vitrification need to be analyzed in the produced embryos.

Table 1. Mean cleavage rate on D3 and blastocyst formation rate on D7, according to different concentration of FCS in the culture media.

GROUPS	CLEAVAGE RATES (%)	BLASTOCYST RATES (%)
G1 - 2.5%	48/67 (61.15)	22/67 (33.35)
G2 - 0%	52/64 (81.73)	22/64 (34.78)
G3 - 0% +2.5%	45/65 (70.12)	18/65 (28.56)
G4 - 0% + 5%	43/63 (70.38)	17/63 (27.71)
G5 - 0% + 10%	45/60 (73.20)	20/60 (32.88)

($P > 0,05$)

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Metabolic status, follicular development and in vitro production of embryos in Nelore heifers fed with different energy levels and fat supplementation

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Introduction

The objective of this study was to investigate the effect of the dietary energy and fat on in vitro production of embryos (IVF) and on the metabolic profile in Nelore heifers (*Bos taurus indicus*).

Material and Methods

Twenty Nelore heifers with average age of 30 months and average weight of 417.0 ± 42.4 kg were used in the experiment. The diet consisted of Brachiaria hay, maize, mineral premix and soybean meal. After two-weeks dietary adaptation and synchronization of the estrous cycle, the heifers were allocated in 3 treatments: 1) T1: animals received 100% of the requirements of energy maintenance (1×M), calculated as NRC; 2) T2: animals received 170% requirements (1.7×M); 3) T3: animals received 170% requirements with addition of 200 g protecting fat (Megalac®). The duration of the treatments was 35 days. After the ovulation, ultrasound examination of ovaries was performed and blood samples were collected daily during 7 days for quantification of FSH, LH, progesterone (P_4) and metabolites. At the end of the period, the oocytes were recovered by in vivo ovum pick-up technique (OPU), and were maturated, fertilized and cultivated in vitro during 7 days. Statistical analysis was performed by using proc GLM, and data are presented as mean ± standard deviation (SD).

Results and Discussion

The daily average weight gain was lower ($P < 0.05$) in T1 (0.1 kg/day) than in T2 and T3 (0.9 kg/day). The serum concentration of albumin, cholesterol, urea and glucose (2.3 mg/dl, 110.8 mg/dl, 22.4 mg/dl and 71.7 mg/dl, respectively) were similar among the groups ($P > 0.05$). The average serum gonadotropins concentration did not vary ($P > 0.05$) between the groups (0.8 ng/ml FSH and 1.4 ng/ml LH). The P_4 concentration was higher in T3 (2.6 ± 1.4 ng/ml), intermediate in T2 (2.1 ± 0.8 ng/ml) and lower in T1 (1.8 ± 0.7 ng/ml; $P < 0.05$). An increase ($P < 0.05$) in the number of small follicles on days 1 and 2 of the estrous cycle was observed in the groups T2 and T3 (T1: 23.2; T2: 34.3; T3: 35.8). However, on day 7 after ovulation, the number of small follicles was similar between the groups ($P>0.05$). Similarly, the total number of medium follicles (4-8 mm) was not modified ($P < 0.05$) by the treatments (average: 4.5). The number of oocytes recuperated by OPU was lower in T1 (7.5 ± 3.2), intermediate in T2 (10.2 ± 6.4) and higher in T3 (11.8 ± 7.3) ($P < 0.05$), and the percentage of embryos developed until blastocysts after 7-days in vitro culture was higher *($P < 0.05$)* for T1 and T2 (35.4% and 38.2%, respectively) than for T3 (26.6%). Our observations suggest that the increase in the energy level and dietary fat supplementation increases the number of follicles and oocytes aspirated by OPU in young Nelore heifers. However, dietary fat supplementation demonstrated no beneficial effect on the embryonic development after in vitro fertilization.

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Effect of oocyte recovery techniques on *in vitro* production of swine embryos

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Introduction

Recent advances in biotechnology have led to the production of cloned and genetically modified pigs by manipulating *in vitro* produced embryos. However, efficiency is extremely low, mainly because of the low developmental competence of manipulated embryos (1). Thus, *in vitro* production of swine embryos must be improved. It is well known that oocytes quality plays an important role on *in vitro* production of embryos (2). The aim of this study was to compare two different techniques to obtain swine oocytes from abattoir ovaries for *in vitro* production of embryos.

Materials and Methods

Ovaries were washed in saline at 35ºC and submitted to slicing (n=240) or aspiration (n=120) simultaneously. In the slicing group, ovaries were held with a hemostatic forceps inside a beaker containing 35mL of Hepes-buffered Tyrode's media (HbT) and follicles (2-6 mm) were incised with a scalpel. Every 5 sliced ovaries, HbT containing follicular fluid was transferred to 50mL centrifuge tubes. In the aspiration group, follicles (2-6 mm) were aspirated using 18 Gauge needle and 5mL syringe. Follicular fluid of each ovary was transferred to 50mL centrifuge tube. Tubes from both techniques were placed in water-bath at 35ºC for 15 min for settling of cumulus-oocyte complexes (COCs). The supernatant was removed and sediment resuspended in HbT and placed in water-bath at 35ºC for an extra 15 min. Sediment was finally resuspended in 15mL of HbT and COCs selected under stereomicroscopy. Oocytes were *in vitro* matured for 44 hours in TCM199 supplemented with 10% porcine follicular fluid and hormones (LH and FSH, only for first 22 hours) at 38.5ºC, 5% CO_2 and high humidity. The oocyte recovery rate of each technique was determined by the ratio between number of selected COCs and ovaries used. Cumulus cells from mature oocytes were mechanically removed prior to *in vitro* fertilization (IVF). For IVF, refrigerated semen was centrifuged on discontinuous Percoll density gradient (45-90%), and the final semen concentration ($5x10^5$ sperm/mL IVF media) adjusted according to motility data. A total of 415 oocytes from slicing group and 115 from aspiration group were fertilized. After 6 hours of IVF at 38.5ºC, 5% CO_2 and high humidity, presumptive zygotes were cultured in Porcine Zygote Media-3 for 7 days under low oxygen tension atmosphere (38.5ºC, 5% CO_2, 5% O_2 and high humidity). Cleavage and blastocyst rates were verified at days 2 and 7 of embryo culture, respectively. Data were analyzed with ANOVA and Tukey test using software Statistica for Windows. A significance level of 5% was considered in all assessments.

Results and Discussion

Oocyte recovery rate (COCs/ovary) was higher for slicing group (2.6 ± 0.3) compared to aspiration group (1.7 ± 0.1; $p < 0.05$). However, no differences on cleavage (10.7 ± 4.8% and 28.7 ± 12.8%, slicing and aspiration, respectively) or blastocyst (24.9 ± 14.8% and 40.1 ± 15.2%, slicing and aspiration, respectively) rates were observed between groups. These results demonstrated that the slicing technique provides a better oocyte recovery rate compared to aspiration of ovaries. In addition, slicing does not affect cleavage and blastocyst rates of *in vitro* production of swine embryos. Considering that *in vitro* production of swine embryos has a lower efficiency when compared to other species, we can conclude that slicing of ovaries is preferred since it provides a higher COC recovery rate without affecting blastocyst production.

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Expression of candidate genes for bovine oocyte competence during follicular development

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Introduction

Developmental competence is defined as the ability of the oocyte to complete maturation, to be fertilized and to undergo normal embryo development and depends on the presence of specific information in the form of mRNA or protein. There is a clear relationship between follicular diameter and oocyte competence, being those obtained from large follicles more developmentally competent in vitro. The aim of this study was to evaluate developmental potential and the expression pattern of candidate genes for the acquisition competence in *Bos indicus* oocytes originated from different follicle diameters.

Materials and Methods

Follicles from slaughterhouse ovaries were dissected, selected and distributed according to their diameter into 4 groups: 1) 1.0 to 3.0 mm; 2) 3.1 to 6.0 mm; 3) 6.1 to 8.0 mm; 4) ≥ 8.1 mm. The cumulus-oocyte-complexes were released by follicle rupture and were classified morphologically, matured, fertilized and cultured in vitro or denuded for diameter measurements and were frozen until gene expression analysis by Q-PCR. The developmental potential of oocytes was evaluated by cleavage and blastocyst rates at 48 h and 168 h post insemination (p.i.), respectively. Data from cleavage and blastocyst rates were analyzed by χ^2 test. The oocyte diameter and relative abundance of mRNA for eight genes (oocyte-specific histone H1 [H1Foo], histone H2A and H3A, stem-loop binding protein [SLBP], growth hormone receptor [GHR], growth differentiation factor 9 [GDF9], bone morphogenetic protein 15 [BMP15] and oocyte-secreted protein 1 [OOSP1]) in oocytes were evaluated by ANOVA and Tukey's test. Non parametric test (Wilcoxon) was used when data were not normally distributed. The results are presented as mean ± SD (oocyte diameter) or SEM (relative abundance of mRNA) and $P<0.05$ was considered statistically different.

Results and Discussion

Oocytes from follicles of 1.0-3.0 mm had smaller diameter than those of larger follicles, with means of 124.1 ± 9.19 µm; 128.0 ± 7.41 µm, 128.2 ± 6.04 µm and 129.3 ± 8.11 µm for oocytes from group 1, 2, 3 and 4, respectively. This result shows that the diameter of oocytes continues to increase until the follicles reach around 3.0 mm even in *Bos indicus* animals, which have smaller size of pre-ovulatory follicle (1). Cleavage and blastocyst rates were higher in oocytes originated from groups 3 (86 and 62%) and 4 (87 and 60%) than those obtained from smaller follicles. Although a higher proportion of cleaved oocytes were observed in oocytes from group 2 (73%) compared to oocytes from group 1 (59%), the blastocyst rate was similar ($P > 0.05$) between them (20% and 34%, respectively). The relative abundance of the H2A transcript rose gradually according to follicle size, being greater in oocyte originated from follicles ≥8.1 mm (1.5 ± 0.21) than those lower than 6.0 mm. The 3.1-6.0 mm follicle size group (1.0 ± 0.17) showed an intermediary expression since mRNA levels were similar either to 1.0-3.0 mm (0.8 ± 0.14) or to 6.1-8.0 mm (1.1 ± 0.24) groups. No changes in transcript level were observed for H1Foo, H3A, SLBP, GHR, GDF9, BMP15 and OOSP1 genes. In conclusion, we confirmed that oocytes from follicles size >6.0 mm are the most competent and that H2A were differentially expressed in those oocytes. These results suggest that H2A has critical implication on oocyte developmental competence. This information contributes towards understanding the mechanism involved in bovine oocyte competence.

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Evaluation of sperm quality parameters to predict in vitro fertility of bovine frozen semen

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Introduction

Individual variation among bulls in the outcome of IVF of bovine embryos has been well reported and the low performance of some bulls has been considered one of the limiting factors in commercial use of IVF. Therefore, the identification of parameters that can predict the in vitro fertility would be very useful to provide information on the performance of a sperm sample, prior to their use in *in vitro* fertilization (IVF). The objective of this study was to evaluate a potential relationship between various sperm parameters and embryo production after IVF.

Material and Methods

Eighty seven semen samples from 10 different bulls were evaluated. In each replicate a 0.5 ml straw was thawed, sperm was evaluated and then deposited in Percoll gradient. After Percoll centrifugation and resuspention, samples were removed again for sperm analysis and the semen was used for IVF. Pre and post Percoll samples were analyzed for motility, sperm morphology (phase contrast), integrity of acrosome (staining with peanut agglutinin [PNA] - fluorescein isothiocyanate [FICT]), integrity of membrane (staining with trypan blue). A total of 2004 oocytes matured in vitro were utilized IVF, and cleavage rate was evaluated on day 2 and blastocysts rate on day 7 after fertilization. The data were analyzed using the program SAS $^{(R)}$ through analysis of correlation and regression.

Results and Discussion

The average of cleavage and blastocysts rates for the ten bulls was 62.9% and 29.3% respectively. The percentage of pre and post Percoll assessments were 37.2% and 67.0% of motility, 82.5% and 91.8% of cells morphologically normal, 36.5% and 51.5% of cells with intact acrosome and 43.5% and 63.4% cells with intact membrane. The results showed an improvement in sperm quality after passage through Percoll. However, very low correlation, varying from 0.21 and -0.17, was observed between all the sperm characteristics evaluated and the rate of cleavage and blastocysts, indicating that these variables are not related. In linear regression analysis, the sperm parameters evaluated before and after Percoll were not able to predict cleavage and blastocysts rates ($P > 0.10$), whether they were considered individually or in combination. These results are in agreement with those reported by Alomar et al. (1) but differed from those obtained by Already Gilan et al. (2), who combining different sperm characteristics found high correlation with fertility. Our results suggest that there is no clear relationship between in vitro fertility and evaluation of sperm viability, measured by different parameters. Therefore, it is possible that the low fertility in vitro may have a multifactorial origin, and it is maybe related with factors other than sperm physical characteristics. However, more studies are needed to confirm this hypothesis.

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Effect of low oxygen tension atmosphere and maturation media supplementation on hsp70 and glutathione content in *in vitro* matured swine oocytes

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Introduction

Studies about *in vitro* production of swine embryos have been important for social, economic and scientific interests. The advances on reproductive biotechnologies (Artificial Insemination – AI; Embryo Transfer – ET and *In Vitro* Production of Embryos – IVP) have enhanced the animal improvement and the productivity in swine, increasing reproductive efficiency and allowing economic viability of swine production. Main problems are insufficient cytoplasmatic maturation of porcine oocytes, high proportion of polyspermic fertilization and low proportion of blastocysts that, in addition, are characterized by a low number of cells[1]. The aim of this study was to evaluate the efficiency of low oxygen tension atmosphere (5% of CO_2; 5% of O_2 and 90% of N_2) on swine oocyte *in vitro* maturation in chemically defined media or supplemented by porcine follicular fluid (PFF) regarding Heat Shock Protein 70 (HSP70) and glutathione (GSH) content.

Materials and Methods

Briefly, oocytes were *in vitro* matured for 44 hours in TCM199 added by 10% of porcine follicular fluid (PFF) or 0.1% of PVA, under low oxygen tension atmosphere or normal oxygen tension atmosphere (5% of CO_2 in air, approximate 20% of O_2). At 0 and 44 hours of maturation, *cumulus oophorus* cells were removed of each group of oocytes. HSP70 content was assessed as described in Kawarsky and King (2001)[2] in order to verify the oxidative stress. GSH content of 25 oocytes was measured by dithionitrobenzoic acid GSSG reductase recycling assay (Glutathione Assay Kit Sigma®).

Results and Discussion

Table 1. Intracellular GSH concentration (pmol/oocyte) of oocytes before (0 hours) and after maturation (44 hours) in different oxygen tension atmospheres and maturation media supplementations.

	Intracellular GSH concentration (pmol/oocyte) (mean ± SD)
0h	2.189 ± 0.426
PFF 20% O_2 (44 h)	2.268 ± 0.974
PFF 5% O_2 (44 h)	1.989 ± 0.799
PVA 20% O_2 (44 h)	2.189 ± 0.921
PVA 5% O_2 (44 h)	2.154 ± 0.969

Different letters represent significant difference ($P < 0.05$).

No differences on GSH content were observed among treatments, indicating that these oocytes have not been capable to store GSH throughout maturation period. No difference at pixels quantification of HSP70 was observed among groups after 44 hours of maturation, PFF 5% O_2 (116.45 ± 40.94^a), PFF 20% O_2 (44.44 ± 12.66^a), PVA 5% O_2 (29.95 ± 7.95^a) and PVA 20% O_2 (58.49 ± 22.2^a), even though these groups were different than 0 hour group (247.41 ± 38.59^b). HSP content decreased throughout *in vitro* maturation of swine oocytes under low and high oxygen tension atmospheres. According to presented data, we conclude that low oxygen tension atmosphere did not affect GSH storage and HSP70 concentrations, regardless maturation media supplementation.

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Superstimulation with eCG or FSH in Holstein (*Bos taurus*) donors inseminated at fixed time

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Introduction
Superstimulation has been successfully done in bovine by using FSH in decreasing or constant doses. However, the high cost of these protocols and the high number of injections has limited a larger widespread of the technique. The present study was performed in order to test the efficiency of a single eCG dose (2000 UI or 2500 UI) instead 8 decreasing dose of FSH (200 mg) in superstimulation protocols for Holstein donors.

Materials and Methods
A total of 12 lactating Holstein cows (*Bos taurus*) were randomly assigned in three experimental groups: eCG-2000 UI, eCG-2500 UI or FSH-200 mg, in a cross-over design. At a random day of the estrous cycle (D0), the animals received a progesterone intravaginal device (1 g P4; DIB®, Intervet/Schering-Plough, Brasil) associated to 2 mg of estradiol benzoate i.m. (RIC-BE®, Syntex, Argentina). From D4, superstimulation was done by a single dose of 2000 or 2500 UI of eCG i.m. (Novormon®, Intervet/Schering-Plough, Brasil), or by 200 mg of FSH i.m. (Folltropin-V®, Bioniche, Canada) administered 8 decreasing doses every 12 h. On D6, 150 µg of $PGF_{2\alpha}$ i.m. (d-cloprostenol; Prolise®, Tecnopec, Brasil) was administered. Thirty-six and 48 h after $PGF_{2\alpha}$, devices were withdrawn and 25 mg of LH (Lutropin-V®, Bioniche, Canada) were administered, respectively. All animals were inseminated once, 16 h after LH. The same semen bath was used in all replicates. Embryo collection was performed on D15. After LH administration, ultrasound examinations were performed during 72 h each 12 h. The statistical analysis was accomplished by proc GLIMMIX of the Statistical Analyses System (SAS).

Results and Discussion
Interactions between replicates were not observed. Therefore, effects of eCG-2000 UI, eCG-2500 UI or FSH-200 mg are summarized in Table 1.

Table 1. Effect of eCG (2000 or 2500 UI) on superstimulatory response of Holstein cows (*Bos taurus*) inseminated at fixed time.

	FSH-200 mg	eCG-2000 UI	eCG-2500 UI	P-value
Number of CL on D15	13.42 ± 1.26^{a}	9.91 ± 1.29^{b}	12.3 ± 1.18^{ab}	0.04
Total of structures	9.58 ± 1.21	7.67 ± 0.98	10.41 ± 0.82	0.10
Transferable embryos	7.92 ± 1.05	6.67 ± 1.07	8.08 ± 0.74	0.35
Frozen/thawed embryos	7.00 ± 1.06	6.33 ± 1.09	6.75 ± 0.51	0.57
Unfertilized structures	0.75 ± 0.13	0.58 ± 0.36	0.25 ± 0.18	0.77
Degenerated embryos	0.91 ± 0.34	0.42 ± 0.19	2.08 ± 0.67	0.56

In the present study, the treatment with eCG did not differ from that with FSH. Treatment with eCG produced a similar number of transferable and freezable embryos compared to treatment with FSH. This result is similar to that obtained by Martins et al (1) in Nelore cows. However, the superstimulation with 2000IU of eCG decrease the number of CL on flushing day (Day 15).The treatment with eCG could be a viable alternative in embryo transfer programs using fixed time artificial insemination in Holstein cows.

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Incidence of apoptosis in bovine embryos produced *in vitro* after prematuration with butirolactone I

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Introduction

The nuclear maturation inhibitor butirolactone I (Bl-I) maintains bovine oocytes at the germinal vesicle stage (GV) in a reversible way. Bl-I can be used in practical situations, as in commercial systems for in vitro production (IVP) of embryos, with the intention of blocking meiosis during the transport of the oocytes from the farm to the laboratory. In this situation, the use of Bl-I would be doubly important: 1) as an attempt of improving oocyte capacitation during transport; 2) in the prevention of oocyte aging during the transport. The aim of this work was to evaluate the effect of Bl-I during a prematuration culture of bovine oocytes on the subsequent embryonic quality and development in vitro.

Materials and Methods

Oocytes (n = 1.391) were pre-matured (pre-IVM) during 24h in basic medium (TCM-199 with 0.2 mM pyruvate, 25 mM sodium bicarbonate and 75 mg/ml gentamicin), according to the following experimental groups: 10 μM Bl-I (B), 10 μM Bl-I + 20 mM HEPES (BH), 100 μM Bl-I + 0.3% BSA (BB); 100 μM Bl-I + 0.3% BSA + 20 mM HEPES (BBH). During pre-IVM, oocytes were cultured in cryogenic vials (15/vial) containing 60 μl of medium recovered with mineral oil, packed in a portable incubator at 38.5°C during the first 5h, and transferred to a incubator at 38.5ºC and 5% CO_2 in air during the 19 remaining hours. After, they were matured at 38.5ºC in atmosphere of 5% CO_2 in air, in basic medium supplemented with 0.6% BSA and hormones (maturation medium) for 24h (control group C1 and C2: just IVM 24 h) or 20h (pre-IVM groups). Oocytes of the second control group (C2) were cultured in maturation medium in the same portable incubator conditions that the pre-IVM groups were cultured. Subsequently, matured oocytes were fertilized and then cultured in SOFm medium with 0.5% BSA and 2.5% FCS in the same temperature and gas atmosphere used for IVM. Cleavage was assessed 48h after insemination (hpi) and embryonic development until blastocyst stage at 192 hpi. Nuclear fragmentation in D8 blastocysts was performed using the TUNEL technique (*In Situ* Cell Death Detection Kit, Fluorescein, Roche Diagnostic Brazil Ltda, São Paulo, Brazil) according to the manufacturer's instructions. Data were analyzed by Tukey's test (P<0.05).

Results and Discussion

Pre-maturation with Bl-I did not affect blastocyst development ($P > 0.05$) (Table 1). Total cell numbers of D8 blastocysts were impaired ($P < 0.05$ *vs* C1) when oocytes were pre-matured with Bl-I (Table 1), but was similar to the C2 group ($P > 0.05$ *vs* C2). We suppose that this result may not be atributed to the Bl-I itself, but to the conditions of culture in the portable incubator, in an attempt to simulate the oocyte transport like in commercial systems for IVP. TUNEL-positive cells in D8 blastocysts was unchanged (Table 1) in all experimental groups ($P > 0.05$ *vs* C1 and C2), with the exception of the BH group ($P < 0.05$). Results shown that, in present experimental conditions, prematuration with Bl-I during oocyte transportation have diminished embryo quality evaluated by the total cell numbers, but the incidence of apoptosis was low. Then, the proposed system of oocyte transport may be a reasonable alternative to maintaining the quality of the oocyte for later use in programs of IVP.

Table1. Embryo development, nuclear fragmentation estimated by the TUNEL technique and total cell numbers in Day 8 blastocysts produced from oocytes matured *in vitro* with 10 and 100μM butyrolactone I. Results are expressed as mean % ± s.e.m., corresponding to seven replicates.

Treatments	Oocytes (n)	Cleaved	Blastocyst D8	Total cell numbers	$TUNEL^+$ cells
C1	392	83.0 ± 3.5 ^{a}	34.4 ± 4.3	95.3 ± 6.2 ^{a} (n=54)	8.2 ± 0.4 ^{b}
C2	207	68.6 ± 6.4 ^{ab}	33.8 ± 5.7	77.0 ± 8.7 ^{ab} (n=40)	10.4 ± 1.0 ^{b}
B	192	67.7 ± 3.5 ^{ab}	36.7 ± 5.2	50.2 ± 3.0 ^{b} (n=28)	14.3 ± 2.5 ^{ab}
BH	201	62.9 ± 5.8 ^{b}	28.3 ± 2.5	57.5 ± 5.8 ^{b} (n=31)	19.1 ± 1.9 ^{a}
BB	196	58.9 ± 5.2 ^{b}	28.3 ± 4.7	63.1 ± 4.2 ^{b} (n=30)	9.5 ± 0.8 ^{b}
BBH	203	57.8 ± 5.0 ^{b}	27.3 ± 3.3	60.0 ± 2.0 ^{b} (n=31)	15.3 ± 1.8 ^{ab}

Different letters within the same column indicate differences among treatments ($P < 0.05$).

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Effect of season and ovarian status on *in vitro* embryo production of domestic cat

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Introduction

The aim of this experiment was to determine the percentage of cleavage and morulae and blastocyst formation derived from oocytes recovered from queen ovaries of three distinct status - follicular, luteal or inactive - during two different reproductive seasons experienced by cats in southeast of Brazil – non breeding season (NBS), comprehending January, February and March; and breeding season (BS), which includes the months of August, September and October.

Materials and Methods

Thirty queens were anesthetized and neutering was performed. Ovaries were classified according to their status and were sliced in PBS supplemented with streptomycin, gentamicin and amphotericin B for cumulus oocyte complex (COC) releasing. Only COC presenting dark and uniform ooplasm surrounded by at least four layers of *cumulus* cells were selected. Grade I COC were washed three times in H-MEM supplemented with 3 mg/mL BSA, 2.0 mM glutamine, 1.0 mM sodium pyruvate, 0.13 mM cystein, 100 mg/mL streptomycin and 100 UI/mL penicillin. Oocytes were incubated in groups of 20-30 in 400 μL of DMEM supplemented with 10μg/mL FSH, 1μg/mL LH, 1μg/mL estradiol, 20 ng/mL IGF-I and 10 ng/mL bFGF (basic fibroblast growth factor) under mineral oil for 30 or 36 hours at 38°C in humidified environment of 5% de O_2, 5% CO_2 and 90% N_2. After 30 to 36 hours, oocytes were fertilized with fresh semen in Ham's F-10 medium supplemented with 3 mg/mL BSA, 0.13 mM de cystein, 1 mM de pyruvate and 100 mg/mL de streptomycin and 100 UI/mL de penicilin (culture medium). Eighteen hours later, the presumptive zygotes were denuded, the percentage of cleavage was determined and every 10 zygotes were transferred to 100 μL drops of culture medium for culture during three days. After 72 hours of culture the percentage of morulae formation was evaluated and these structures were transferred to drops of the same culture medium. At the eighth day of culture blastocyst formation was analyzed. Statistic differences between groups were analyzed through ANOVA.

Results

The results are presented in Table 1 and 2.

Table 1. Absolute and relative frequency of cleavage, morulae and blastocyst formation derived from oocytes recovered from queen ovaries of three distinct status - follicular, luteal or inactive - and rate during non breeding season.

	Inactive (%)	Luteal (%)	Follicular (%)
Total of fertilized	272	162	134
Cleaved	67 (24.63) [a]	35 (21.6) [a]	33 (24.62) [a]
Morulae	45 (16.54) [b]	21 (12.96) [b]	22 (16.41) [b]
Blastocyst	22 (8.09) [c]	14 (8.64) [c]	11 (8.21) [c]

Table 2. Absolute and relative frequency of cleavage, morulae and blastocyst formation derived from oocytes recovered from queen ovaries of three distinct status - follicular, luteal or inactive - and rate during breeding season.

	Inactive (%)	Luteal (%)	Follicular (%)
Total of fertilized	102	198	86
Cleaved	66 (64.7) [a]	127 (64.14) [a]	55 (63.95) [a]
Morulae	42 (41.17) [b]	80 (40.41) [b]	34 (39.54) [b]
Blastocyst	24 (23.53) [c]	47 (23.73) [c]	21 (24.41) [c]

Discussion

The results of this experiment demonstrate that no statistic difference was verified in the frequency of cleaved embryos and morulae and blastocyst formation when comparing the three ovarian conditions in the same season. However, the breeding season presented better results considering cleavage and morulae and blastocyst formation.

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Embryonic and early fetal development in Miniature ponies

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Introduction

In recent years, the population of Miniature equids has expanded worldwide. Miniature mares are a potential model for comparative reproductive studies with larger equine breeds and among species as evidenced by the results of recent studies on ovarian, uterine, and hormone dynamics during the estrous cycle. Descriptive and comparative studies of early pregnancy are important for advancement of basic knowledge and may have practical implications in understanding early pregnancy loss. The purpose of this study was to evaluate the main characteristics and events that occur during embryonic and early fetal development in Miniature ponies (n = 13) until Day 60 (ovulation = Day 0).

Materials and Methods

Evaluations were carried out by daily examinations with transrectal palpation and ultrasonography after Day 6. Location changes of the vesicle within the uterus were evaluated by daily mobility trials consisting of an examination every 10 min for two consecutive hours (13 location determinations per trial) from Day 10 until the day of fixation (cessation of mobility).

Results and Discussion

The first day of detection of an embryonic vesicle was Day 11.5 ± 1.3 (mean ± SD) by ultrasound and Day 17.2 ± 1.5 by transrectal palpation. A period of extensive intrauterine mobility, as previously reported for horses, ponies, and jennies, was observed from Days 10 to 15. During the mobility phase and before the daily two-hour trial, the embryonic vesicle was detected more often ($P < 0.0001$) in the right horn (57%) than in the left horn (19%) and uterine body (24%). During the mobility trials, the vesicle was more often ($P < 0.0001$) in the uterine horn ipsilateral (333/684; 49%) than contralateral (201/684; 29%) to the corpus luteum (CL). Fixation of the vesicle occurred on mean Day 15.8 ± 1.5 at a diameter of 21.4 ± 4.2 mm. More vesicles fixed in the right (62%) than left (8%) uterine horn ($P < 0.004$). The additional vesicles (30%) appeared to fix in the uterine body. More ovulations (67%) occurred on the right side (previously reported as significant for the estrous cycle). There was a close temporal relationship between expansion of the vesicle, increase in uterine tone, and fixation of the vesicle. The mean diameter of the embryonic vesicle or conceptus was 6, 25, 31, 47, 58, and 63 mm on Days 11, 20, 30, 40, 50, and 60, respectively. The growth curve of the conceptus had a distinct plateau between Days 18 and 25. The conceptus expanded at a daily rate of 3 mm before the plateau and 1 mm after the plateau. The conceptus remained spherical up to approximately Day 17, but thereafter dramatic changes occurred in the shape (e.g., oblong, triangle, irregular). The embryo proper was first detected on Day 20.5 ± 1.6 on the ventral aspect of the vesicle and opposite to the mesometrial attachment. The embryo gradually moved toward the mesometrial attachment. The allantoic sac was first detected between Days 24 and 29 (26.5 ± 1.4) and occupied approximately 10, 50, and 90% of the conceptus on Days 27, 33, and 40, respectively. The umbilical cord was first detected on Day 41.0 ± 1.9, coinciding with the beginning of the descent of the fetus, and 92% of the fetuses were at the ventral pole of the vesicle by Day 53. The mean crown-rump length of the embryo proper/fetus was 5, 13, 19, 28, and 40 mm on Days 20, 30, 40, 50, and 60, respectively. A solitary accessory CL was detected between Days 47-50 in only 4 (31%) of the Miniature ponies; no additional CLs were detected before Day 60. Results indicated that the diameter of the embryonic vesicle and size of embryo proper and early fetus were similar to what has been reported in larger breeds despite extreme differences in body size. Characteristics in the Miniature ponies that appeared to differ from the reported results in larger breeds were as follows: 1) ovulation more often from the right ovary; 2) embryonic vesicle more often in the right uterine horn or ipsilateral to the CL during the mobility phase; 3) fixation more frequent on the right side; and 4) incidence of accessory CLs greatly reduced.

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Nuclear maturation in gas mixture atmosphere of vitrified immature equine oocytes *in vitro*: influence of the *cumulus oophorus* cells

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Introduction

Frozen-thawed equine oocytes have a reduced rate of maturation, when compared with unfrozen controls (1). Immature oocytes would be more resistant to cryopreservation because the mitotic fuse would not have been formed at this stage (2). Oocytes in more advanced stages of maturation would be more sensitive to freezing; the mitotic fuse is disorganized during the MII and due to the increased polyploidy during the fertilization (3). The objective of this study was to evaluate nuclear maturation of vitrified immature equine oocytes *in vitro*, and the effect of the characteristics of the *cumulus oophorus* cells.

Materials and Methods

Equine oocytes were collected from slaughterhouse ovaries. Immature oocytes were vitrified-thawed (OPS method) and then matured *in vitro*. In the control group, unfrozen oocytes were matured *in vitro*. The solution used included: 2.8 M formamide, 2.7 M ethylene glycol, 2.8 M DMSO, 7% polyvinylpyrrolidone and 1% X-1000. For thawing, the OPS were placed in air for 5 sec and the contents of straws were emptied into 1M sucrose solution. The maturation was conduced in TCM199 + 400 ng/ml eGH + 200 ng/ml IGF-I + 0.1% BSA + 100 UI/ml penicillin + 50 μg/ml streptomycin, on a gas mixture (CO_2 5%, O_2 5% and N_2 90%). After maturation during 30 h, the oocytes were stained in Hoechst 33342 for evaluating of nuclear maturation in fluorescence microscope.

Results and Discussion

The protocols tested in the present study were efficient to vitrify immature equine oocytes in the presence of *cumulus oophurus* cells, as no difference was observed in maturation rates after vitrification/re-warning (Table 1). When compared against other studies dealing with vitrification of immature equine oocytes (9), the rates obtained in the present study would be higher. Logistic regression analysis did not identify differences in oocyte maturation as function of joint effects of vitrification and characteristics of the *cumulus oophurus* (Table 2).

Table 1. Maturation rates for fresh equine oocytes submitted to distinct vitrification treatments, according to the characteristics of the oocyte-*cummulus* morphology.

	CP oocytes				EX oocytes				Total			
	Immature		MII		Immature		MII		Immature		MII	
	n	%	n	%	n	%	n	%	n	%	n	%
Control	58	70.7	24	29.3	37	46.8	42	53.2	95	59.0	66	41.0
Vitrified	58	78.4	16	21.6	38	54.3	32	45.7	96	66.7	48	33.3

Table 2. Risk of equine oocyte maturation as a function of the interaction between vitrification and characteristics of the *cumulus oophurus* cells

	CP oocytes [a]			EX oocytes [b]			CP+EX oocytes [c]		
	OR	95% CI	P	OR	95% CI	P	OR	95% CI	P
Control	-	-	-	-	-	-	-	-	-
Vitrified	0.67	0.32; 1.38	0.2758	0.74	0.39; 1,41	0.3644	0.72	0.45; 1.15	0.1679

[a]Constant: -0,88; Deviation: 266,65; Model DF: 230 (P = 0,048); [b]Constant: 0,13; Deviation: 305,54; Model DF: 218 (P = 0,570); [c]Constant: -0,36; Deviation: 599,52; Model DF: 451 (P = 0,023); *Considered as the reference level in all three models

In conclusion, immature equine oocytes can be *in vitro* matured after vitrification. The oocyte-*cummulus* morphology, at the time of oocyte collection, does not influence the nuclear maturation of vitrified immature equine oocytes.

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Comparative study on the development of renal systems in bovine and bubaline embryos during the early gestation period

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Introduction

According to Dyce et al (2004; 1) bovine kidneys maintain the same structure than fetal ones; that means multi-lobar and multi-pyramidal structure, Bovine and Bubaline are two different species but both of them are classified as large ruminants, having many similarities in morphological aspects. Some differences, especially on reproductive aspects, may be noted: bovine has a gestation period of 285 days while bubaline has a period of 315 days. The present study has the objective to describe morphological aspects of the development of bovine and bubaline renal system during the embryonic period of 10 to 50 days.

Material and Methods

Embryos were collected in slaughterhouses and had been measured and photographed using the Crow-Rump method. For morphological description they had been fixed with Bouin solution for light microscopy and with glutaraldehyde 2,5% for transmission electron microscopy.

Results and Discussion

In bovine and bubaline embryos within the gestational period of 14 to 20 days the presence of pronephro was not observed, however it was found the mesonephro, starting in the middle region of the embryo, containing innumerous tubules of two distinctive types, one with simple cubical epithelium, and the other with brush borders. According to Bacha (2000; 2) the first ones to appear in the adult animal are the collecting tubules, and the second the proximal convoluted tubules. These structures gave us morphological indications of functional activity. Bovine mesonephro showed innumerable mitochondria and interdigitations in the interior of its tubular cells, indicating high ionic transport activity. It was observed that the metanephro, or definitive kidney, initiates its development in embryos within the gestational period of 23/24 days in both species. Those findings indicate that the involution of the mesonephro occurred simultaneously to metanephrogenic differentiation and that it starts from the cranial portion of the transitory organ onwards. The only structures remaining from the mesonephron are the mesonephric tubules that will be part of the efferent ducts in male testis (Browder et al 1991; 3). In 30/35 days buffalo embryos, glomeruli conformation and macula densa were found as Browder et al (1991; 3) described; in bovine ones with 25/28 days this structure was defined as a cellular array that possibly represent glomeruli structures At the beginning of the fetal phase, within the 45/46 days of the gestational period, the metanephro already exhibited filter units (nephrons), with its respective glomeruli, proximal and distal convoluted tubules and the Loop of Henle. This is an ongoing study, so our partial conclusions about bovine and bubaline renal systems development are related to the absence of pronephro tissue, at 14 to 20 days of gestation, and that the degenerative process of the mesonephro starts simultaneously to metanephrogenic differentiation. Bubaline with 30/35 days have glomeruli and mesonephro`s tubules, these last ones being similar to collecting and convoluted tubules. Despite some differences of days, the development of renal system in bovine and bubaline embryos is very similar. We hope that those findings could be used in ultrasound and other image techniques as a diagnostic tool to determine abnormalities on the bubaline and bovine embryos renal development.

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Influence of body condition previous to a short undernutrition period on embryo survival in ewes

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Introduction

Short periods of undernutrition are usual in Mediterranean breeds of sheep, mainly due either to the variation of food availability or to physiological conditions throughout the productive period of ewes. It is well known that both long-term and short-term changes of the diets and body condition affect fertility in the ruminants, particularly trough changes of the ovary function (1). Thus, undernutrition has shown negative effects on follicle dynamics, oocyte quality and embryo survival (2, 3). The aim of this study was to evaluate the effect of body condition (BC) prior to a short subnutrition on ovulatory follicle characteristics and embryo survival in ewes during the breeding season.

Materials and Methods

Adult and cycling Rasa Aragonesa ewes maintained at the experimental farm of University of Zaragoza, Spain, during the breeding season were assigned into two groups according their mean BC score (BC3, high, n = 20, mean BC 3.00 ± 0.04; and BC2, low, n = 18, mean BC 2.18 ± 0.05; $P < 0.001$), receiving a diet to maintain a constant BC in each group. After 45 days, both groups were synchronized with intravaginal pessaries and randomly divided into two groups and fed to provide 1.5 (Control, C) or 0.5 (Low, L) times daily maintenance requirements. Therefore, ewes were divided into 4 groups: C3 (BC3+1.5 M diet), L3 (BC3+0.5 M diet), C2 (BC2+1.5 M diet) and L2 (BC2+0.5 M diet). Ovulatory follicles were evaluated from Day -3 to ovulation by ultrasonography. At estrus (Day 0) ewes were mated and on Day 5 embryos were recovered by mid-ventral laparotomy and classified according to their developmental stage and morphology. After that, embryos were incubated to evaluate their *in vitro* development during 48 h. Live weight and BC were measured every 15 days and throughout the experimental period.

Results and Discussion

All ewes maintained BC during the whole experimental period (BC3 = 2.80 ± 0.05 and BC2 = 2.22 ± 0.08). Only ewes fed with L diets (L3 and L2) experienced a reduction in live weight. Ovulation rate, number of recovered ova and fertilized embryos per ewe were not affected by level of nutrition (Table 1). However, a higher BC seems to override the detrimental effects of undernutrition on ovulation rate ($P < 0.1$), viability rate ($P < 0.1$) and in vitro development of embryos ($P < 0.1$) (Table 1). A weak relationship was found between the diameter of ovulatory follicles at ovulation time and the in vitro embryo development ($r = 0.804$; $P = 0.08$). These results seem to indicate that a good BC score prior to undernutrition period could be a determinant factor to improve embryo survival and development during early pregnancy stages in undernourished ewes.

Table 1. Ovarian response and embryo production in Rasa Aragonesa ewes with two different mean body condition (BC=3 and BC=2) and fed with two different level of nutrition: 1.5 M (C) and 0.5M (L) times daily maintenance requirements during the breeding season

	C3	L3	C2	L2
No. of ewes in estrus	9/10	10/10	9/9	8/9
Mean diameter of ovulatory follicle (mm)	5.7 ± 2.8 [a]	5.4 ± 2.6 [a]	5.7 ± 2.9 [a]	5.0 ± 2.9 [b]
Ovulation rate	1.8 ± 0.3	2.2 ± 0.3 [c]	1.7 ± 0.3	1.5 ± 0.3 [d]
No. of recovered and fertilized embryos*	1.2 ± 0.2	0.8 ± 0.2	1.3 ± 0.3	1.1 ± 0.3
No. of viable embryos	1.0 ± 0.3	0.7 ± 0.3	1.0 ± 0.3	0.6 ± 0.3
Viability rate (%)	83.3	83.3	66.7	50.0
Rate of *in vitro* development of embryos	75.0	75.0 [c]	61.1	35.7 [d]
Pregnancy rate (%)**	55.6 (5/9)	50.0 (5/10)	66.7 (6/9)	50 (4/8)

*All recovered embryos were fertilized in all groups. **Percentage of ewes with viable embryos on Day 5 after estrus. Different superscript letters in the same row indicates $P< 0.05$ (a, b) or $P<0.1$ (c, d).

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Effect of different levels of protein supplementation on collection and development *in vitro* of intact and perforated rabbits (*Oryctolagus cuniculus*) embryos

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Introduction
Nutrition is an important factor in the success of embryo transfer (ET), highlighting the levels of vitamin A, crude protein, fat and energy. The objective was to evaluate the efficiency of supplementation of different protein levels on collection and development *in vitro* of intact and perforated rabbit embryos.

Materials and Methods
Thirty-three New Zealand White does were used in the summer (n1) and autumn (n2) to evaluate two types of commercial rations with 13 (T13; n1 = 7, n2 = 8) e 16% (T16; n1 = 8, n2 = 10) of crude protein. The animals were weighted at the beginning (BWi), weekly to adjust the feed intake to 3.5% BW and at the end (BWf) of the experiment. The embryos collections were performed 72 hours after mating. Before *in* vitro culture, half of the grade I and II embryos from the treatments were perforated in the mucus layer to the zona pellucidae by a needle of 0.3 µm. The embryos micromanipulation were done in a PBS drop and cultured in synthetic oviduct fluid (SOF) medium, in an incubator at 38.5°C, with 5% CO_2 and saturated moisture by 24 hours. The embryos were classified pre and post-culture for morphology and quality. To evaluate the effect of the ration on animal development and embryo classification pre-culture, the season's weather was considered as a block (DBC). To evaluate the embryo classification after culture, the treatments were considered in a factorial design 2x2 (levels of protein and type of embryos - intact and perforated ones) in DBC. The development data were analysed by the Anova and the embryo classification by logystic regression. All data were analysed by the SAS 8.0 program at 5% probability.

Results and Discussion
The animals were equally assigned to the treatments T13 and T16, according to the mean BWi (3.82 and 3.87 kg, respectively) and showed no statistical difference between the means of BWf (4.16 and 4.05 kg) or between the dry matter intake (134.51 and 135.44 g/day). There was a statistical difference between treatments for the average daily gain (5.04 and 2.62 g/day) and feed efficiency (35.47 and 19.40). Embryos numbers (6.27 and 6.06 embryos) and the morphological classification does were similar, but different from (1). The levels of protein did not influence the embryos evaluation classified into grades I, II and III, but the T13 rabbits had more degenerated embryos. There was no ($P > 0.05$) interaction between treatment and embryo for embryonic classification by the morphology. The embryonic micromanipulation did not affect morphological classification ($P > 0.05$). The embryos from T13 animals and the intact showed better development post-culture, changing from morula stage to the initial blastocyst. So, the commercial diet with 13% of protein showed a better performance and the *in vitro* culture of intact embryos has better development.

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Health indicators and reproductive biomarker responses in the sentinel fish *Astyanas fasciatus* from the Furnas Reservoir, Brazil

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Introduction

In Brazil, the environmental ecotoxicology has attracted recent interest from the scientific community, mainly in the South and Southeast, but there is a shortage of native species to be used as bioindicators and parameters suitable to act as physiological biomarkers. Anthropogenic activities have caused profound alterations in physical and chemical characteristics of the aquatic environment. The Furnas hydroelectric power lake, the largest reservoir of the Southeastern Brazil, has been subject to environmental pollution. Dejects of domestic and industrial sewage *in natura*, contamination by agrotoxics and use of the water for the tourism are preoccupant to the public institutions (1). Recently, the lambari *Astyanax fasciatus* (Cuvier, 1819) an abundant species in the Furnas reservoir, has been identified as a potential bioindicator of environmental impact to rivers and lakes in South America (2). The aim of this study was analyzing the health indicators and the main parameters of the macro and microscopic reproductive biology of *A. fasciatus* in Furnas reservoir.

Material and Methods

For this study a total of 583 fishes were caught in 2006 in five sampling points on Furnas reservoir: Turvo (reference site) with little anthropogenic interference, and four impacted sites. The fishes were caught in the lake margins close to the cities of Barranco Alto, Fama, Boa Esperança and Guapé. The specimens were preserved in formalin 10% for obtaining biometric data and for analyses of the biological indices. Samples from liver and gonads were fixed in Bouin's fluid and processed for histology. Morphometrical analyses of the gonadal maturation and follicular atresia were established using light microscope. The water quality in each site was assessed through chemical analyses of the main parameters: temperature, oxygen, turbidity, pH, electrical conductivity and heavy metals.

Results and Discussion

The tools for assessing the environmental impact on the health indicators and reproductive biology of *A. fasciatus* used in this study are of high ecological significance and reflect the impacts accumulated during years of anthropic activity over the Furnas reservoir. *A. fasciatus* reproduces the whole year in the Furnas reservoir, has fractional spawning, showing tolerance to environmental pollution. In the recipient sites of untreated domestic sewage (Boa Esperança and Guapé), the load of organic material reduced the water quality leading to disturbances in sexual proportion. Intersex fish of *A. fasciatus* were detected in these sites, indicative of endocrine disruption. In Guapé was also observed increase in the levels of follicular atresia. In Fama, which receives industrial waste from Varginha city, it was detected fallings of growth and gonad maturation and a decrease of vitellogenic oocytes during the breeding peak. The contamination of water, especially by heavy metals can be considered the main cause of the last alterations (3). The results sustain *A. fasciatus* as bioindicator of environmental impact in the Furnas reservoir. In addition, the physiological and reproductive biomarkers examined in this study should be used as parameters to assess environmental impact in the reservoir, establish measures of preservation and management, aiming to conservation of the ecosystem and maintenance of its icthyofauna.

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Immunolocalization of caspase-3 and HSP70 during embryogenesis of *Prochilodus lineatus*

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Introduction

Programmed cell death for apoptosis is an important process in normal development of many organ systems and tissues. During morphogenesis, cells that are no longer required are removed by apoptosis (1). The key apoptotic factors are a cascade of cysteine proteases known as caspases that are widely expressed during embryogenesis. Caspase 3 is an effector molecule that activates the target proteins of the intrinsic and extrinsic pathways of the apoptosis. These proteins of the apoptotic routes are responsible by nuclear shrinking, cleavage of cytoskeletal proteins and activate kinases involved in the membrane blebbing (2). Different external and intrinsic apoptotic stimuli induce the accumulation in the cells of a set of proteins known as heat shock proteins (HSPs). These proteins play an essential role as molecular chaperones by preventing the protein aggregation (3). The stress response triggers rapid induction of HSPs, resulting in thermo-tolerance while increasing levels of stress result in the apoptotic response (4). In the present study we used immunohistochemistry to localize caspase-3 and HSP-70 during embryo development of the *P. lineatus*, a fish species found in southeastern Brazil and considered a very important fishery resource.

Material and Methods

The embryos were obtained at the Furnas Hydrobiology and Hatchery Station, through artificial breeding by hormonal induction. After fertilization, the eggs were incubated in vertical incubators with a controlled temperature (26ºC). Embryos were collected in several periods of development since 1 h post-fertilization (2-4 blastomeres) until hatching. They were fixed in Bouin's fluid, embedded in glycol methacrylate and stained with toluidine blue to identify morphological events of the development and apoptosis. Samples of embryos in stages of cleavage, blastula, gastrula, segmentation and hatching were fixed in methanol/DMSO, embedded in paraplast, sectioned and immunostained with antibodies anti- caspase-3 and HSP-70.

Results and Discussion

Embryos are highly susceptible to stress including thermal shock at early cleavages stages and acquire increased resistance as they progress through the blastula and gastrula stages. Caspase-3 immunostaining associated with morphological features such as chromatin condensation, cytoplasmic retraction and cell fragmentation in apoptotic bodies was observed in cells indicating apoptosis after blastula stage. HSP70 was constitutively expressed during embryogenesis in the cytoplasm of all blastomeres, increasing the intensity in later embryogenesis stages, when apoptosis was also increased. In the gastrula, the yolk syncytial layer presented no labeling for caspase-3, however, it was strongly stained for HSP70. Cells in apoptosis were numerous during development of the nervous tissue after segmentation. These results suggest that HSP70 could play a role in the modulation of apoptosis during embryo development in *P. lineatus*.

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Glycogen related to placenta and fetus of shorned or not shorned sheep during the gestation

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Introduction

In Brazil, the production of sheep is a factor of great economic and social importance in the southern region. One of the main problems are the reproductive losses, caused by the low weight at birth and the low temperatures encountered during the time of birth. Three factors that regulate the growth in fetal sheep and determine the weight at birth were the maternal nutrition, the size of the placenta and pre-delivery. Jones and Desoye (1993) studied one phenomena that happens in human beings: diabetic pregnancy women have children with significant increase of the weight at the time of born. Sheeps have their conceptuses with a significant increase of the weight when they are submitted to shear process. Based on these data we propose to study the glucose, as a source of energy in most animals that can be stored in the form of glycoprotein. These ones were analyzed in placenta, liver and bone tissues of fetuses and lambs born from ewes shorn (G1) at around 70 days of gestation, compared with a control group (not unshorn animals (G2), in Southern Brazil.

Material and Methods

Our data were obtained from samples collected from thirteen animals, six from sheared group, and seven not sheared. Placentome samples were collected, subjected to macroscopic examination (weight, number, thickness, width, and length) and fixed (Gendre fluid), processed and stained for light microscopy (HE) and histochemistry (PAS) examination (Fig 1A-D). Images were analyzed using the 400 Kontron KS 3.0â program in a 60 Axioscopyo BX Zeiss microscope, coupled to an Axioplan Zeiss camera (Fig 1C-G). Five random areas were selected in each plate for measurement and comparison of glycogen contents.

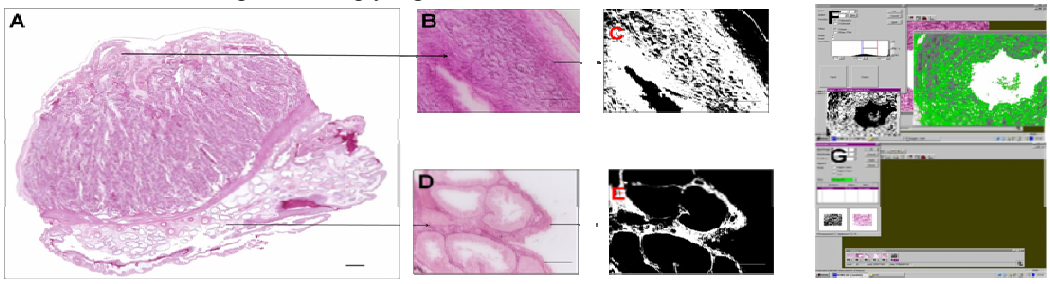

Fig 1 Methodology of the measurement of glycogen deposits in A – ovine placentome PAS, 200µm; on B and D na example of the sheatory areas that were done the measurements, PAS, 25µm; on C and E the quantified area or white color; 25 µm ; on F and G the especif computer program used for this procedure of glycogen areas quantification.

Results and Discussion

Our results showed that glycogen concentrations (in placenta) and fetal weights were 36.1% and 11.9% higher in G1 than G2, respectively ($P < 0.05$). Placentome areas with higher glycogen concentrations were at the connective tissue and fetal vessels. The macroscopic results indicated that the treatment of shorn not significantly increased the fetal and placental weights, and so little measures regarding placentomes. The microscopic analysis, shows that the average between the areas of glycogen quantified in placentome were not different ($P < 0.05$). Already in the liver at the portal triad region and in the rectus femoris muscle were found a significant increase of glycogen deposits ($P < 0.05$) (Table 1).

Table 1: Results (Mean ± SD) of analysis of glycoprotein deposits on different tissues.

Tissue	Placentome		Portal vein - liver		Rectus femularis muscle	
Group	G1	G2	G1	G2	G1	G2
Values in µm^2	39712^{NS}	31235^{NS}	54499.23^{*}	34830.73^{*}	41128.7^{*}	31942.7^{*}

$P < 0.05$. NS - no significance.

Conclusion

With this results we can conclude that the shorn done at 70 days of gestational period influence on the accumulation of glycogen in the liver and muscle of the fetuses and thus increase the weight of them at birth.

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In vitro nuclear maturation of compact and expanded equine oocytes treated with roscovitine

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Introduction
Due to the distance between equine slaughterhouses and the research laboratories it is been of great interest to delay the beginning of nuclear maturation (MII) of oocytes used for ICSI or NT using the roscovitine, a specific kinase inhibitors which induces a reversible block of meiosis. The objective of this experiment was to evaluate the effect of roscovitine treatment for 24 h in equine oocytes with cumulus classified as compact or expanded.

Materials and Methods
Oocytes were obtained from slaughterhouse ovaries and distributed in: compact = both the cumulus and the mural granulosa cell with no signs of expansion, or expanded = presence of expanded mural granulosa cells and/or cumulus cells. Control oocytes were matured *in vitro* (IVM) for 30 h in HTF:BME (1:1) media with 0,3 % BSA, IGF-1, EGF, eGH, eFSH, estradiol and gentamicin. Roscovite treated oocytes were incubated in TCM199 media with 10% FBS, 66μM roscovitine and gentamicin for 24 h before IVM. Three replicates were made with 20 oocytes/group/replicate in 5% CO_2 in air at 38.5°C. Nuclear maturation in both groups was evaluated with Hoechst 33342 after 30 h of IVM. Statistic differences were analyzed through Chi-square test. For samples ≤5 Fisher test was used ($p < 0.05$).

Results and Discussion
In the control groups statistic differences were observed between MII ($p = 0.036$; 17.6% and 30.% for compact and expanded oocytes, respectively) and degenerated oocytes ($p = 0.0006$; 45.8% and 23.3% for compact and expanded, respectively). However, when the roscovitine was used, differences were no longer seen. When groups were compared, a statistic tendency ($p= 0.0581$) appeared between MII (30.7% and 17.6%) and degenerated ($p = 0.0580$; 30.7% and 45.8%) compact oocytes treated and non treated with roscovitine . These results confirm the superiority in meiotic competence of equine expanded oocytes (1 and 2). The results also demonstrate the efficiency and reversibility of the roscovitine treatment (3), specially for the compact oocyte group which presented a higher MII and a lower degeneration rate (4 and 5). This effect may be linked with a synchronization of cytoplasmic/nuclear maturation in these oocytes during the incubation time.

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Comparison of three methods for superovulation of brown brocket deer (*Mazama gouazoubira*)

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Introduction

The knowledge of assisted reproduction techniques for wild animals should be useful for future *in situ* and *ex situ* conservation programs. Therefore, it is essential to know not only reproductive biology of the target species, but also the best way to manipulate its reproductive cycles. This project aims to establish a superovulation protocol for brown brocket deer (*Mazama gouazoubira*), by evaluating the ovulation rate, the presence of functional corpora lutea, the number of embryos and their development stages after superovulation using different methods.

Materials and Methods

Six female *Mazama gouazoubira* received an intravaginal device containing 0.33 g of progesterone (ovine and caprine CIDR® - Pfizer®) for 8 days, followed by 0.5 mg injection of oestradiol benzoate (Estrogin® - Farmavet Produtos Veterinários® Ltda) at the time of insertion and 265 µg of cloprostenol (Ciosin® - Shering Plough Coopers®) at the time of removal. Afterwards, the hinds were divided into 3 groups (n = 2): the first received an intramuscular injection of 600IU PMSG (Folligon®1000 - Intervet International BV®), on the fourth day after insertion of CIDR® (Treatment A), the second an intramuscular injection of 300IU PMSG, at the same time (Treatment B) and the third an intramuscular injection of 250IU FSH (Pluset® - Calier®) dissolved in PVP (MW = 40,000; 30% solution - Sigma®), also at the same time (Treatment C). A fertile male was utilized for estrus detection and fertilization, every 6 hours, after the treatments until the end of behavioral estrus. During 28 days feces were collected daily and later analyzed for concentrations of progestagen and estrogen (ELISA). The corpora lutea and unruptured follicles (over 4 mm) were counted and the embryos were collected via laparoscopy, seven days after the first copulation. All the hinds received an intramuscular injection of 265 µg of cloprostenol 14 days after CIDR® removal and 30 days after this last injection the treatments were crossed over (all the animals went through all 3 Treatments). All data were expressed as the mean ±SEM. Analysis was conducted using MINITAB® Release 14 Software. P-values <0.05 were considered significantly different.

Results and discussion

There were five hinds in Treatment B, because one of the hinds died. The mean ovulation rate in Treatment A (3.40 ± 0.62) was significantly higher than the mean ovulation rates for Treatments B (0.8 ± 0.49) and C (0.67 ± 0.42). However, the superovulation response was not significantly different between Treatment groups (5/6 hinds; 2/5 hinds; and 2/6 hinds; in A, B and C, respectively). The presence of large follicles (over 4mm) was also detected along with corpora lutea in superovulated animals so that the total ovarian stimulation in Treatment A (4.17 ± 1.05) was significantly different from Treatment C (1.33 ± 0.49) and not different from Treatment B (1.83 ± 0.37). There was no significant difference for initial time and duration of behavioral estrus and the mean duration of inter-luteal phase. However, the mean progesterone concentration in the inter-luteal phase for Treatment A (1,013.09 ± 177.87 ng/g feces) and B (988.61 ± 196.67 ng/g feces), were significantly different from Treatment C (411.12 ± 17.51 ng/g feces). There was no significant difference in estrogen concentration. The mean progesterone concentration produced by animals that presented visible corpora lutea was not significantly different (6,277.94 ± 2,232.47 ng/g feces; 2,970.15 ± 564.38 ng/g feces and 1,196.79 ± 117.75 ng/g feces; in Treatments A, B and C, respectively). On the other hand, the mean diameter of corpora lutea in Treatment A (7.33 ± 1.31 mm) was significantly different than in Treatment B (3.94 ± 0.19 mm) and C (2.18 ± 0.49 mm). No embryo was collected. Thus, although Treatment A presented the greatest mean ovulation rates and total ovarian stimulus, it cannot be said that an appropriate protocol was established for superovulation induction and embryo sampling of *Mazama gouazoubira*. Total ovarian stimulus was considered low (1) and no embryo was collected. However, we should also consider problems with the embryo collection technique. This experiment shows the difficulty in adapting known protocols in domestic species and highlights the need for specific studies, which better adjust to reproductive biology of the species and make the use of reproductive biotechniques as a tool in wild species conservation possible.

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Effects of hCG application on the recovery rates and morphology of eqüine *cumulus oophorus* complex obtained from immature follicles

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Introduction

In mares, the *ovum pick up* (OPU) of immature follicles results in poor recovery rates. This fact seems to be linked with the presence of a thecal pad beneath the *cumulus* cells, which act as an anchor for the *cumulus* attachment. In preovulatory follicles (>35mm), final oocyte maturation is accompanied by the expansion of the *cumulus* cells, which releases the oocyte from the follicular wall. Oocyte recovery rate with OPU in preovulatory follicles (>35 mm) increases to until 77% after hCG administration (1), while in immature follicles (<35 mm) a much lower recovery rate is achieved (6.9% to 52%) (2). The objective of this experiment was to evaluate the recovery rates and the morphology of *cumulus oophorus* complex (COCs) obtained via OPU in equine immature follicles from mares stimulated with EPE + hCG.

Material and Methods

The follicular dynamic was followed daily in ten crossbreed cycling mares, rotated in group 1: EPE/hCG and group 2: control. Both treatments started with the application of 250 μg of cloprostenol (1,5 ml Lutalyse® - Pfizer Saúde Animal) seven days after ovulation. To the group EPE/hCG it was administered EPE (25 mg, i.m., twice daily) until the most of follicles reached 22-27 mm, when hCG (Vetecor, 2500 UI, i.v., Calier do Brazil) was then administered. The OPU sessions were performed 24 h after hCG administration in the EPE/hCG group or after the largest follicle reached 30 mm in the control group. All follicles presenting more than 15 mm were aspirated. The fluid collected from each individual follicle was examined in a stereomicroscope (40X) for visualization of oocytes and visual analysis of granulosa cells. Follicular fluid was collected at the moment of aspiration to progesterone analysis, performed by radioimmunoassay (RIA) using the commercial Kit Coat-A-Count® Progesterone (DPC, Los Angeles, California.). For comparison purposes some samples were collected from preovulatory follicles (> 35 mm) of mares treated with EPE + hCG, according to the protocol described in Group 1. For the ultrastructural analysis, 7 oocytes from EPE/hCG and 5 from the control group were fixed in 2.5% glutaraldehide with phosphate buffer (0.1M, pH 7.4) for 24 hours and processed according to a standard technique. The ultra thin cuts were obtained with a diamond knife, mounted on cooper grids and stained with uranil acetate and lead citrate. The samples were analyzed on a transmission electron microscope (Philips CM 100).

Results and Discussion

According to the results obtained the EPE with hCG was not capable of inducing an increase in oocyte recovery rate from immature follicles(15,5 % oocytes/follicle in EPE/hCG vs 16,7% in control; $p > 0.05$), although a higher recovery rate per mare was observed (70% in EPE/hCG x 50% in control). However, the treatment with EPE/hCG was efficient in inducing precocious granulosa cells expansion and increasing intrafollicular progesterone concentration. The number of follicles presenting expanded granulosa cells was significantly higher ($p < 0.05$) in the EPE/hCG (29/45 follicles; 64.4%) group compared to the control group (1/30 follicle; 3.3%), and the P_4 concentrations were similar between preovulatory follicles and immature follicles treated with EPE/hCG (268.57 ng/m ± 55.91 vs 158.80 ng/ml ± 29.62, respectively) and higher than what was observed in non-treated (control) mares (82.05 ng/ml ± 13.85) (Tukey's test; $p<0.05$). In this experiment, oocytes from both groups presented a great amount of vesicles containing an electron dense material characterized as cortical granules, with different sizes and electron-density and distributed in clusters into the cytoplasm. Moreover, junctional complexes were still seen between oocyte and granulosa cells in both groups. This results indicate that oocytes from follicles smaller than 35 mm may need a longer time to respond the hCG stimulus inducing the lost of contact between the oocyte and the surrounding granulosa cells. In conclusion, the superovulatory treatment with EPE associated with hCG was able to induce some of the follicular modifications characteristic of the preovulatory stage. However, the process was not completed since oocyte cytoplasmic maturation and recovery were not improved.

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In vitro embryo production (IVEP) from fertility-impaired beef cows through the use of different oocyte harvesting methods

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Introduction

In vitro embryo production (IVEP) is the only technique at the disposal of assisted reproduction laboratories which may enable fertility-impaired cows to continue breeding. There are basically two ways of approaching *in vitro* embryo production from fertility-impaired donors. The first method would be the slaughter of the animals to retrieve oocytes from the ovaries post slaughter and the second is to apply ultrasound guided oocyte aspiration (OPU) for a period before slaughter (1). The aim of this study was thus to compare the two IVEP approaches and a combination thereof in terms of effectiveness in producing embryos.

Material and Methods

Donor cows (n = 40), (various beef breeds and parities) of superior genetic quality and with impaired fertility were presented to the reproduction laboratory. All donor cows were screened for a period of 2 to 3 weeks by means of the ultrasound scanning procedure to access follicular activity. Some animals (n = 20) were subjected to prolonged ultrasound guided oocyte aspiration protocols (an average of 16.75 OPU sessions per donor, ranging in number from 5-38) in order to obtain oocytes for IVEP procedures. This procedure (OPU) was continued until no more follicles developed or no more embryos could be produced from a specific donor animal. Other animals (n = 20) were slaughtered immediately. All donors were super stimulated before slaughter by inserting a progestagen ear implant (Crestar, Intervet, Holland) for 5 days followed by a single administration of 2500 IU eCG (Folligon, Intervet, Holland) and slaughtered 2.5 days after eCG administration. The results were thus recorded for the following contemporaneous groups of animals or combinations thereof: Group 1 (period of OPU) (n = 20); Group 2 (slaughter after OPU – same animals as in Group 1) (n = 20); Group 3 (combination of Group 1 and 2) (n = 20) and Group 4 (slaughter without any OPU) (n = 20). Statistical analysis was performed by means of a one way analysis of variance (ANOVA) and probability values <0.05 were considered to be statistically significant.

Results and Discussion

The results are depicted in Table 1. A direct comparison between Group 3 and 4 indicates an increase (+10) in the mean number of embryos produced per donor cow when OPU is combined with slaughter. Although not statistically significant, it was significant in practical terms and justifies the use of this advanced and labour intensive OPU procedure to be applied on superior donor cows. The same tendency in obtaining a higher embryo output from OPU-derived material, compared to abattoir material has been recorded in Buffalo cows (2).

Table 1. Mean (±SE) number of oocytes recovered, embryos produced, embryo production success rate and embryo quality per treatment group over the total period.

Group	Number of oocytes recovered	Number of embryos produced	Embryo production (%) (success rate)	Embryo grading (%)		
				Grade 1	Grade 2	Grade 3
1	24.7 ± 9.4^{a}	6.6 ± 5.4^{a}	22.6 ± 16.8^{a}	46.0 ± 36.3^{a}	41.5 ± 38.3^{a}	12.6 ± 19.0^{a}
2	73.0 ± 38.3^{b}	10.7 ± 15.7^{a}	14.5 ± 15.6^{a}	28.5 ± 32.8^{a}	51.8 ± 40.5^{a}	19.8 ± 37.1^{a}
3	97.7 ± 40.2^{c}	17.2 ± 16.6^{a}	17.4 ± 14.7^{a}	46.2 ± 26.8^{a}	42.8 ± 23.5^{a}	11.1 ± 10.4^{a}
4	61.8 ± 40.3^{d}	7.2 ± 8.5^{a}	10.1 ± 9.6^{a}	36.0 ± 42.2^{a}	46.8 ± 41.1^{a}	17.1 ± 31.4^{a}

[a-b-c-d] Values within a column with different superscripts differ significantly (P<0.05)

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Influence of flunixin meglumine at the time of embryo transfer on pregnancy rates of recipient cows

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Introduction

The usage of advanced techniques such as artificial insemination (AI) and embryo transfer (ET) programs results in extraordinary improvement in genetic merit of dairy and beef cows. However, pregnancy rates associated with ET are generally lower than those using AI. The manipulation of the cow reproductive tract, during embryo transfer, has been shown to increase release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) into the uterine lumen (1, 2). Several studies have shown negative effects of $PGF_{2\alpha}$ on embryonic survival in beef cows (3, 4). Moreover, addition of $PGF_{2\alpha}$ to the culture medium has been shown to inhibit in vitro development of bovine embryos (5). The objective of this study was to determine whether administration of the prostaglandin inhibitor, flunixin meglumine (FM), immediately prior to embryo transfer would increase pregnancy rates in recipient cows.

Materials and Methods

Estrus (day = 0) was synchronized in crossbred cows (n = 184) by giving one injection of $PGF_{2\alpha}$ (500 µg of cloprostenol, i.m.; Ciosin®, Schering-Plough, Brazil) and a single in vitro produced embryo was transferred 6 to 8 days after estrus (day = 6-8). At the time of embryo transfer, animals were randomly assigned to receive either 1.1 mg/Kg of flunixin meglumine (i.m.; Flunixin Injetável®, Chemitec Agro-Veterinária, Brazil) (FM; n = 92) or remain untreated (control; n = 92). Data collected at transfer included stage of embryo development, embryo quality and ease of transfer score (1-3; 1 = gun easily manipulated to the site of transfer, 3 = difficult). Pregnancy results were obtained 30 and 60 days following transfer by ultrasonography. The logistics procedures and chi-square analysis of SAS were used for data analysis.

Results and Discussion

Overall pregnancy rates did not differ ($P > 0.05$) between recipients receiving flunixin meglumine (44.6 %; 41/92) versus control recipients (40.2%; 37/92). Stage of embryo development, embryo quality or ease of transfer score did not present any effect on pregnancy rates following embryo transfer ($P > 0.05$). Although not monitored in this study, there are preliminary evidences supporting the idea that manipulation of the cervix and uterus when performing embryo transfer does induce the release of $PGF_{2\alpha}$ from the uterus (1, 2). It is possible that cervical and uterine manipulation in these recipient cows did not result in significant production or release of $PGF_{2\alpha}$ from the reproductive tract since the vast majority of the embryos were reported as being very easily transferred into the upper third of the uterine horn. In conclusion, administration of flunixin meglumine, a $PGF_{2\alpha}$ synthesis inhibitor, at the time of embryo transfer did not increase the rate of pregnancy establishment in recipient cows under the conditions utilized in this experiment. Therefore, administration of flunixin meglumine to embryo recipient cows at the moment of embryo transfer may not be recommended.

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Pregnancy rate of beef cows with fixed timed artificial insemination using different doses of GnRH analog

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Introduction

Looking forward to intensifying the adoption of reproductive biotechnologies, the hormonetherapy has been used as method for estrus synchronization and on fixed time artificial insemination (FTAI), in the sense of reducing the need of hand work for observation of estrus and application of AI. GnRH associated with Prostaglandin (Pg) synchronizes the growth of ovarian follicles and the growing follicle can be ovulated in a presided way by using of a second dose of GnRH 1.5 to 2.0 days after the application of Pg. This protocol was been called Ovsynch and presents results similar to the insemination with natural estrus (40% of pregnancy) in lactating cows (2). The objective of this study was to evaluate the IA with fixed time using different doses of GnRH analog, as well its economic impact on the pregnancy rate of beef zebu cows.

Material and Methods

A total of 88 nursing multiparous crossbreed beef cows, with mean post-partum period of 120 days and body condition score ranging from 2 to 3 (scale 1 to 5), were randomly distributed in 2 groups: G1 (n = 45) and G2 (n=43), receiving, respectively, 100 and 200 μg of GnRH analog in the FTAI. Twenty five days later the cows were put together to 4 bulls up to the end of time of gestation checking (around 40 days after FTAI), performed by ultrasonography in real time.

Results and Discussion

Pregnancy rates in FTAI were 26.6% (12/45) and 20.9% (9/43), respectively, for G1 and G2 ($P > 0.05$). For the period using cleaning up bulls, the pregnancy results were 24.4% (11/45) and 25.5% (11/43), for G1 and G2 ($P > 0.05$), respectively (Table 1). The cost of medicines, acquired in the local market, corresponded to US$2/ml of GnRH, (Profertil®) and US$1.3/ml of Prostaglandin (Prostaglandin®), summing US$5.31 for G1 and US$9.31 for G2. The registered pregnancy rates in the present study were similar to those recorded by Ahuja et al. (1), working with lactating cows, in anestrous, with low BCS (21% of pregnancy rate for the Ovsynch protocol). The pregnancy rates after FTAI with Ovsynch protocol in crossbreed Nelore cows, using 100 or 200 μg of GnRH analog can be considered satisfactory. The improvement in the pregnancy rate can be obtained by using cleanup bulls and reduction in price of medicines by using smaller doses of GnRH analog.

Table 1. Pregnancy rates of beef cows with fixed time artificial insemination (FTAI) and with cleanup bulls.

Treatment	N	Pregnancy (with FTAI)	Pregnancy (With cleanup bulls)	Total Pregnancy Rate (FTAI + Cleanup bulls)
G1	45	12 (26.6%)	11 (24.4%)	23 (51.1%)
G2	43	09 (20.9%)	11 (25.5%)	20 (46.5%)
Total	88	21 (23.8%)	22 (25.0%)	43 (48.8%)

$P > 0.05$.

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Immunological response of Nelore cows superovulated with pFSH injected by subcutaneous route

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Introduction

Conventionally, the superovulation induction of embryos donors' cows is done by intramuscular route (im) using decreasing doses of pFSH, twice a day, for 4 days. The unique injection of pFSH, subcutaneous (sc) can also induces a superovulation response, with results very close to the ones obtained with several injections im. Considering that the application subcutaneous results in a slow absorption of the hormone (from porcine origem), it can induce immunological response, which can turns the animal refraction to the next injection. This study aimed to evaluate the antibody production, by the superovulated cows, against pFSH, during two superovulation series, sc route.

Materials and Methods

Fourteen Nelore cows showing good body condition (score 3 and 4, in a scale from 1 to 5) , were used. Folicular growth was done with progestagene (CIDR-B, InterAg, New Zealand) and injection of oestradiol benzoate 2.5 mg (Estrogin, Farmavet). In the day 4th , seven animals received a sc injection of 400 UI of pFSH (Pluset, Lab. Calier, Spain) applied in the animal shoulder cover. The other 7 animals, received im injections for 3 days, twice a day. Next morning of day 7th, a intramuscular injection was done with 150 mcg of cloprostenol (Veteglan, Lab. Calier, Spain) and the CIDR-B removed in the afternoon. Animals were artificially inseminated 48 and 72 hours after Veteglan injection. Ovarian response (corporea lutea- CL) and the embryo collection were done 7 days after insemination (1). Such procedure was done again 60 days after collection. Blood samples from jugular vein were taken before each superovulation procedure and 30 days after second superovulation. Sera samples from the cows were kept frozen. Sera anti-pFSH was done in rabbits, with pFSH conjugate (BSA/Carbodiimide; 2). To verify antibodies against pFSH, a sandwich ELISA was performed, using the HRP-TMB enzymatic system. Calibration curve was used to establish sensibility of immunoassays to 0.1 unit of pFSH por mL of sera R = 0,949. Data was submitted to F test and correlation analysis. **Results and Discussion**

60 days after first superovulation, from seven Nelore cows superovulated sc, four cows in the SC treatment presented antibodies, measured in O.D. at 420 nm, with variation of 0.01 to 0.052. In the second superovulation procedure, 120 days, response showed a variation of 0.178 to 0.292 in the O.D. at 420 nm, in six animals. Apparently the presence of antibodies did not affect the ovarian response or the embryos production after second superovulation procedure. Correlation between CL and immunological response was 0.2445 and between immunological response and viable embryo number was 0.0695. The F test ($P<0.05$), table 1 showed no differences. The im superovulated 7 cows did not showed immune response at measurable level. We can conclude the presence of anti-pFSH antibodies, in a lower level than O.D. 0.292 does not interfere on the embryos production.

Table 1. Antibody production (AC), CL and embryos produced by each animal (V1-V7) after 120 days of superovulation procedure.

AC	0.294	0.179	0.314	0.359	0.292	0.01	0.223	0.229
CL	13	6	8	12	9	11	12	10.14
viable embryos	8	0	5	0	8	8	7	5.14
total embryos	9	5	6	3	9	11	14	8.14

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Factors Associated with Successful of Laparoscopic Artificial Insemination in Turkish Native Fat Tailed Sheep

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Introduction
An important factor determining the efficacy of insemination is the appropriate timing of semen deposition with respect to ovulation, which is influenced by the method used for estrus synchronization (1). Fertilizable life of frozen–thawed spermatozoa is often half that of fresh spermatozoa thus pregnancy rate could be lower in ewes inseminated with frozen–thawed semen. Anel et al., (2) have reported that one of the most important factors influencing fertility after artificial insemination was season. In this study, it was investigated the effects of factors such as different estrus synchronization programs, season of the insemination and origin of the semen (fresh or frozen) on success of laparoscopic artificial insemination in native Turkish fat tailed breeds.

Material and Methods
In the first trial, an orally active progestagen, melengestrol acetate (MGA 0, 25 mg/head/day; n = 72), vaginal progestagen sponges; 30mg fluorogeston acetate (FGA; n = 38), and 60mg Medroxyprogesteron Acetate (MAP; n = 180) used for estrus synchronization (12 days) in the breeding season (September-November). In the second part of the trial, fresh (n = 41) or frozen (n = 45) semen were used in intrauterine insemination of ewes synchronized with FGA (30 mg) in the breeding season. In the last trial, laparoscopic artificial insemination carried out in the breeding (September-November) (n = 120) or out of the breeding season (May-July; n = 40). Ewes synchronized with synthetic progestagen treatment were injected i.m. 500 I.U. PMSG at the end of each synchronization programs. In all trials, concentration of semen was 100 mil spermatozoa per 0.5 ml and intrauterine inseminations carried out at 55 h after the end of synchronization treatment. Lambing data were analyzed by binomial logistic regression.

Results and Discussion
Similar lambing rates were obtained with fresh (63.4%) and frozen (48.9%) semen while lambing rate of FGA (60.5%) and MAP (71.1%) treated ewes were higher ($P < 0.01$) than ewes treated with MGA (33%). Lambing rates were similar in breeding (64.3%) and out of season (52.5%) inseminations. In conclusion, the most important factor effecting laparoscopic insemination is determined as estrus synchronization methods.

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Service period of Gyr (*Bos taurus indicus)* cows presenting two follicular size class and submitted to progesterone protocol during early postpartum period[1]

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Introduction

Progesterone and estradiol protocols has been used to induce first estrus and ovulation in postpartum and to reduce service period (SP) in suckled postpartum anestrous zebu cows (1), mainly in animals presenting larger dominant follicles (2, 3). The objective of this study was to investigate the service period and the pregnancy rate of postpartum anestrous Gyr cows submitted to progesterone and estradiol protocol, according to two follicular size class.

Material and Methods

One hundred anoestrus Gyr cows (62.5 ± 20.8 days postpartum, body condition score 3.39 ± 0.41 - scale 1 to 5) were allocated in four treatments (T1, T2, T3 and T4, n = 25) according to the diameter (mm) of the largest follicle present in ovaries at first ultrasound evaluation (day 0). Animals from T1 and T2 presented large follicle diameter less than 10 mm, and T3 and T4 follicle diameter greater than or equal to 10 mm. Cows in T2 and T4 were administered (day 0) with intravaginal progesterone-releasing devices (1.9g of progesterone, CIDR® - Pfizer, Brazil) at the time of 1.0 mg estradiol benzoate (Estrogin®- Farmavet, Brazil). Following CIDR removal (day 7), all cows from T2 and T4 were given an intramuscular injection of 1.0 mg of estradiol cipionate (ECP® - Pfizer). Estrus detection was twice daily (07:00 and 18:00h) and artificial insemination performed 12h after estrous, and the pregnancy diagnose carried out 30 days after insemination.

Results and Discussion

Ovulation rates differ ($p < 0.05$) and pregnancy rates did not differ ($p > 0.05$) between treatments (table 1). The length of service period was short (76.37 ± 30.99 days; $p < 0.05$) for group of animals (T4) presenting larger (≥10mm) dominant follicle and that were submitted to CIDR protocol. However, cows (T2) presenting small (≤10mm) dominant follicle and receiving CIDR had similar service period of no treated animals (T1 and T3), independent of the diameter of the dominant follicle. Shortening of service period in postpartum anestrous Gyr cows treated with progesterone + estradiol is similar to other study (4) with heavier live weight dairy cows receiving norgestomet ear implant. There was interaction ($p < 0.05$) of dominant follicle diameter and CIDR treatment on shortening the service period and reproductive efficiency of Gyr cows.

Table 1. Pregnancy rates and service period of anestrous Gyr cows administered with CIDR device, according to two follicular size class.

Treatment	N	Ovulation Rate	Pregnancy Rate	Service Period (days)
T1	25	12% (3)[a]	32% (8)[a]	130,12 ± 31,43[A]
T2	25	60% (15)[b]	36% (9)[a]	107,3 ± 37,14[A]
T3	25	28% (7)[a]	48% (12) [a]	109,91 ± 37,86[A]
T4	25	88% (22)[c]	64% (16)[a]	76,37 ± 30,99[B]

[a,b]Different letters within columns are statistically different ($p < 0.05$) by Fisher test.
[A,B]Different letters within columns are statistically different ($p < 0.05$) by "t" test.

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Induction of estrus in dogs with an injectable formulation of deslorelin

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Introduction

The control of the estrous cycle in bitches, mainly the induction and synchronization of estrus and ovulation, is fundamental for the application of assisted reproduction techniques. The development of an efficient and predictive method of estrus induction is an important step in clinical approach of infertility in the bitch, and should be useful for commercial breeding, resulting in a greater frequency of births (1). However, the lack of effective protocols is still an obstacle for the use of reproductive biotechnology in dogs. GnRH agonists have shown potential for inducing a fertile estrus in bitches. The deslorelin, a synthetic GnRH agonist, presents potency and stability many times larger than native GnRH. Recently, an injectable short action formulation of deslorelin was developed, tested firstly in mares, which raised interest in the study of estrus induction also in bitches. Based in the literature and in the potential of GnRH agonists to induce estrus in bitches, the present study has the purpose of evaluating the effect of an injectable formulation of deslorelin on the reproductive system of anestrous bitches, testing one or multiple applications.

Materials and Methods

Thirteen sexually mature mongrel bitches were used. The previous estrous cycles were not known and the animals were initially characterized in anestrus by vaginal cytology, with predominance of parabasal cells, and by plasmatic concentration of progesterone below 0,5ng/mL. The bitches were randomly divided in three groups, as follow: G1 (n = 5, single IM injection of 2,0mg of deslorelin), G2 (n = 5, four IM injections of 2,0mg of deslorelin in alternate days) and Control Group (n = 3, four IM saline injections in alternate days). Clinical evaluation, sexual behavior, vaginal cytology and plasma progesterone concentration were daily assessed. Ovaryhysterectomy and macroscopic evaluation of the uterus and ovaries were done on the 25th day of diestrus post treatment in the bitches which responded and at 45 days in the remaining.

Results and Discussion

The results are summarized in Table 1. In G1, none of the bitches showed signs of estrus, two animals developed bloody vulvar discharge, vulvar edema, attraction to the male and vaginal smears with predominance of erythrocytes, intermediate and few superficial cells, compatible with proestrus. In G2, all animals presented proestrus, four presented estrus and three ovulated, resulting in functional corpora lutea, and high progesterone concentration until day 25 of diestrus, when ovaryhysterectomy was performed. In Control Group one bitch showed signs of proestrus at the end of the experiment, probably unrelated with any bioestimulation mechanism. The duration of the stages of deslorelin induced cycles and the progesterone profile were similar to previously reported in spontaneous (2) and GnRH induced cycles (3), and no side effects were observed. Deslorelin injectable formulation was considered effective to induce estrus in anestrous bitches, with multiple injections. Further studies are needed to determine the appropriate dosage and duration of treatment.

Table 1. Induction of estrus and ovulation rates in mongrel dogs treated with an injectable formulation of 2,0mg of deslorelin, in one (G1) or multiple applications (G2). Days from beginning of treatment (mean$^{\pm}$s.d.) and percentage of incidence within each group.

Responses	G1 (n=5)	G2 (n=5)	Control Group (n=3)
Proestrus	4.5 ± 2.1 (40%)	5.6 ± 1.5 (100%)	35 (33%)
Estrus	-	13.3 ± 1.5 (80%)	-
Ovulation	-	16.0 ± 2.0 (60%)	-
Luteal phase	-	19.3 ± 1.5 (60%)	-

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Coherent light incidence in semen: an optical approach to evaluate spermatozoa surviving

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Introduction

Sperm viability is a phenomenon routinely used to evaluate the quality of sperm samples, which consist of many attributes, such as structural and physiological characteristics, in addition to motility (M) parameters. There are many approaches to conduct the motility analysis, one of them being the biospeckle laser technique (BSL). Illumination of sperm samples by laser has been a potential approach to evaluate the motility pattern of ejaculates, once the interference images formed, which are known as dynamic speckle or biospeckle patterns, can reproduce the activity of the sample under illumination (1). One way to grade the motility is to seek its behavior throughout the time, therefore, correlating the viable cells with motility decrease (2). This work presented the evaluation of motility over the time of bull's sperm samples using the BSL.

Material and Methods

Frozen sperm cells of six bulls were analyzed, divided in two groups, according to the kinetic pattern (I – M $\geq$ 50%, n = 3; e II – M < 50%, n = 3). Straws with 0.5 ml of semen, under a concentration around 30 to 35 millions of cells, were thawed at a temperature of 37° C during 60 seconds, and monitored by light microscopy. Each sample, after the thawing process, was illuminated successively six times, with a time rate of two minutes among the illuminations, at room temperature. During this process the samples were also monitored by light microscopy.

Results and Discussion

An index was proposed to grade the motility (M) and the velocity (V) obtained by light microscopy evaluation [IND = (V x 20 + M) / 2], and then compared to the BSL index, known as inertial moment (IM). The results yielded regression coefficients adjusted to IM and IND (r^2 = 70.50%; r^2 = 95.28%) to groups I and II, respectively. The relations between IM and IND in time can be described by the equations Y = -78.68 + 4.88X and Y = 2,530.80-188.78X + 4.76X^2-0.04X^3, to groups I and II, respectively. BSL technique followed the expected behavior of the samples over the time showing its ability to be considered as an alternative method to sperm kinetic analysis.

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Determination of *in vitro* progesterone diffusion coefficient in a polymeric blend by Fick´s second law

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Introduction

In FTAI protocols, progesterone (P_4) sustained-releasing devices are required. These devices are often constituted of a nylon skeleton covered by a mixture of P_4 and silicone or P_4 and polymeric blend (1). The mathematical modeling for the drug release process offers relevant data concerning mass transfer, as well as it allows the analysis of parameters and variables such as device geometry, initial concentration and distribution, and also drug releasing mechanism. In order to achieve a better understanding of the kinetics of P_4 release, its diffusion coefficient was calculated following Fick´s second law, represented by the equation: $\frac{\partial q}{\partial t} = D_e \frac{1}{r} \frac{\partial}{\partial} \left(r \frac{\partial q}{\partial r} \right)$. These data allow the prediction of P_4 concentration needed in a polymeric blend.

Materials and Methods

Two biopolymers-based devices were used in this study, both with 147 cm^2 of superficial area: DISP8 contained 46% of poli-hidroxibutirate and valerate (PHB-V), 46% poli-ε-capolactone (PCL) and 8% P_4 (n = 4) and DISP10 contained 45% PHB-V, 45% PCL, 10% P_4 (n = 4). Experiments were conducted in a drug dissolutor under USP 23 (2) specifications. An alcohol / water (60 / 40) mixture was used as diffusion medium. Medium samples were collected after 2 min, 2, 4, 8, 12, 24, 48, 60, 72, 84 and 96 h. P_4 concentrations were analyzed by spectrophotometer in a 244 nm wavelength. P_4 diffusion coefficient was determined by the "golden search" method and the following function: $F_{obj} = \sum_{j=1}^{n} \left(m_{lib_j}^{MOD} - m_{lib_j}^{EXP} \right)^2$, where n is the number of experimental data, $m_{lib_j}^{EXP}$ the P_4 experimental mass released to medium (mg) and $m_{lib_j}^{MOD}$ the P_4 mass released to medium obtained with the model (mg).

Results and Discussion

Diffusion coefficient values were 1.12 x 10^{-8} (cm^2/s) for DISP8 device and 1.36 x 10^{-8} (cm^2/s) for DISP10 device. Both devices have the same geometry and differ only in the polymeric matrix and P_4 concentration. Diffusion coefficient obtained ranged from 0.5 - 22 x 10^{-8} cm^2/s, what was also found by Valenzuela-Calahorro et al. (2003) (3) when determining P_4 diffusion properties in four carbon-based materials.

Conclusion

It was observed that the diffusion equation can adequately represent the P_4 release by the devices DISP8 (R_2 = 98.5) and DISP10 (R_2 = 98.2).

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Characteristics of follicular development during synchronization of nulliparous buffaloes during unfavourable reproductive season in the northeast of Brazil

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Introduction

The brazilian buffalo milk industry has increased in the last years what demands a uniform production all year long to attend the increasing market. Despite of in the northeast of Brazil buffaloes are not markedly influenced by seasonal conditions changes, the heifers always present some seasonality. The aim of this experiment was to compare the follicular development characteristics of nulliparous buffaloes during the summer submitted to two intravaginal device progesterone protocols.

Materials and Methods

This study was done in a dairy buffalo farm located at the district of Taipu-RN, Brazil Latitude -05° 37' 18"and Longitude 35° 35' 48" during February 2007 for the CIDR protocol (1.9 g of progesterone) and February 2008 for the PRIMER protocol (1.0 g of progesterone). The buffaloes were treated on Day 0 with an intravaginal progesterone-releasing insert (CIDR or PRIMER) plus 100 mg of GnRH. On Day 7 all buffaloes received 500UI of eCG and on Day 8, 0.150 mg of PGF2α. On day 10, ovulation was induced with 100 mg of GnRH. All buffaloes had 3.5 score of body condition based in a scale from 1 (thin) to 5 (fat). Follicular dynamics was monitored by transrectal ultrasound (Pie Medical, linear transducer of 7.5 MHz) every day from device insertion until device removal and every 6 hours after device removal until ovulation. Follicular diameters were obtained from the average of two frozen images. All female buffaloes were put together with a vasectomised teaser bull fitted with a chin-ball marking harness to detect the estrus behavior.

Results and Discussion

There was no statistical difference between progesterone treatments (1.9 g vs. 1.0 g). Follicle diameters data are shown in Table 1. Other authors (1) have reported the influence of progesterone over follicular development. The use of devices which presents less progesterone can contribute for the development of the follicles promoting better ovulating rates during the unfavorable reproductive season of buffaloes.

Table 1. Data (mean ± s.e.m., when applicable) of follicular dynamics during the treatment with progesterone associated to GnRH and eCG for synchronization of follicular wave and ovulation in female buffaloes.

Analyzed variables	Treatment	
	CIDR	PRIMER
Percentage of animals ovulating after first GnRH injection	12.5% (1/8)	25.5% (2/8)
Percentage of animals with start of a new follicular wave	100% (8/8)	100% (8/8)
Interval from device removal to estrus behaviour	67.0 ± 6.0 (hours)	65.0 ± 4.0 (hours)
Diameter of dominant follicle at Day 0	7.1 ± 0.6 (mm)	7.6 ± 0.6 (mm)
Diameter of ovulatory follicle at second GnRH injection	10.5 ± 1.1 (mm)	10.8 ± 0.7 (mm)
Rate growth of the ovulatory follicle	1.5 ± 0.1 (mm/day)	.6 ± 0.2 (mm/day)

P > 0.05 within rows for all end points.

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Tracking of ovary response by transrectal ultrasonography following estrus synchronization treatment of Santa Ines ewes

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Introduction

Ovarian evaluation by ultrasound (US) has permitted advances in the comprehension of reproductive physiological phenomena (2) contributing to the understanding of estrus synchronization protocols and induction of ovulation procedures as well as superovulation (3). Once ovarian and endocrine data are scarce for Santa Ines ewes in literature, this study had the objective to evaluate ovarian response, by transrectal US, following estrus synchronization treatment and the progesterone profile of ewes raised extensively in the southeast of the state of Bahia, northeast of Brazil.

Materials and Methods

The experiment was made in a farm located in Macarani-Bahia latitude 15°18'14''S and 15°12'204''W from April to May/2008. Seven non-lactating ewes, aging between 24 and 36 months and presenting body condition score 3.5 in a scale of 1 to 5 (1 = thin and 5 = fat) were used. All ewes received mineral salt ad libitum and had their estrus synchronized with intravaginal sponges containing 60 mg of medroxyprogesterone (MAP - Progespon, Shering-Plough, Brazil) for a period of 14 days, associated with 250 IU of eCG – Novormon, Schering-Plough, Brazil) given by intramuscular injection at the moment of sponge removal. Twelve hours after sponge removal, the females were observed for estrus behavior every 6 hours with the help of a vasectomized teaser with a chin ball. The second estrus following treatment was monitored twice daily by transrectal US during the experiment. The ovaries were scanned daily for a period of 20 days **(**Pie Medical 100 LC-8.0 MHz). Blood samples were collected daily by jugular vein puncture for progesterone assay. It was used a Kit IMMULITE2000 (DPC, USA) with sensibility of 0.19 ng/ml and CV intra and inter assay of 9% and 11%, respectively.

Results and Discussion

It was possible to visualize both ovaries in (6/7) in 85.7% of ewes. During the scans, near the estrus, spherical anechoic structures measuring approximately 5 mm in diameter were visualized indicating the presence of preovulatory follicles. At day 4 after estrus, it was observed echogenic spherical structures with delimited edges, which were classified as corpus luteum. Progesterone concentrations during the follicular phase and luteal phase were 0.36 ± 0.06 ng/ml and 3.43 ± 0.33 ng/ml, respectively. In one ewe 1/7 (14.3%) it was possible to define a corpus luteum of low echogenicity. This condition was confirmed by irregular and low progesterone serum concentration during the estrous cycle (1). The transrectal US made possible the evaluation of ovarian response to estrus synchronization treatment as cited in literature (2, 3). The data presented herein shows that the Santa Ines ewes, when submitted to estrus synchronization, present similar results for progesterone concentrations as reported for ewes of temperate climate.

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Effect of FSH treatment at intravaginal progesterone device withdrawal on pregnancy rate of crossbred heifers (*Bos taurus* x *Bos indicus*) submitted to FTET

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Introduction

An approach to increase the overall pregnancy rate is giving eCG (during the estrus synchronization protocol) to induce single and multiple ovulations (1). A study with a lower dose of eCG (400IU) resulted in only 3/156 cows with two CL, but plasma progesterone (P4) concentrations and pregnancy rates were higher than in untreated controls (2). Two studies were performed to evaluate the effect of FSH treatment in synchronization of ovulation protocols on pregnancy rate of crossbred heifers (*Bos taurus* x *Bos indicus*) submitted to fixed-time embryo transfer (FTET). The experimental hypothesis was that FSH had similar efficiency than eCG when administered at the time of intravaginal progesterone device withdrawal.

Material and Methods

Only cyclic heifers (n = 541) with body condition score ≥3 (1 to 5 scales) were used in both experiments. In a random day of the estral cycle (Day 0), all animals received an injection of 2mg of estradiol benzoate (RIC-BE®, Tecnopec, Brazil) and an intravaginal P4 device (PRIMER®, Tecnopec, Brazil). At day 8, the device was removed, 150µg of d-Cloprostenol (Prolise®, Tecnopec, Brazil) and 0,5mg of Estradiol cypionate were administered in all animals. At this moment, the heifers were homogenously allocated in one of two (Experiment 1; eCG Group and FSH10 Group) or three (Experiment 2; eCG Group, FSH10 Group and FSH20 Group) experimental groups. At Experiment 1 (n = 265), the animals of eCG Group (control) received 400 IU of eCG (Novormon®, Syntex, Argentina) and the heifers of FSH10 Group received 10µg of FSH (Folltropin-V®, Bioniche, Canada). At Experiment 2 (n = 276), the same groups were used and one more group was included: FSH20 Group. The animals of this group were injected with 20µg of FSH on day 8. The FTET was performed by only one veterinary on day 17 and all embryos used at these experiments were produced *in vitro*. The pregnancy diagnosis was performed by ultrasonographic examination 23 days after ET.

Results and Discussion

Conception and pregnancy rates and the number of heifers transferable/treated are presented in Table 1 and 2. Other experiment (Experiment 2) was performed with one more group using a higher dose of FSH (20µg), to verify if the results with this dosage would be similar to eCG results. These data suggest that the dosage of 20µg of FSH has similar efficiency to 400IU of eCG when used in synchronization of ovulation protocols for FTET in crossbred heifers (*Bos taurus* x *Bos indicus*).

Table 1. Conception rate, pregnancy rate and number of heifers transferable/treated in Experiment 1.

	eCG	FSH10	P Value
Conception rate (%)	42.3 (55/130)	40.0 (38/95)	< 0.05
Pregnancy rate (%)	37.4 (55/147)	32.2 (38/118)	< 0.05
Transferable/treated (%)	88.4 (130/147)	80.5 (95/118)	0.07

Table 2. Conception rate, pregnancy rate and number of heifers transferable/treated in Experiment 2.

	eCG	FSH10	FSH20	P Value
Conception rate (%)	38.4 (33/86)	39.7 (29/73)	40.3 (29/72)	< 0.05
Pregnancy rate (%)	34.7 (33/95)	30.5 (29/95)	33.7(29/86)	< 0.05
Transferable/treated (%)	90.5[a] (86/95)	76.8[b] (73/95)	83.7[a] (72/86)	0.046

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Effect of injectable mineral supplementation (Multimin) on pregnancy rate of crossbreed heifers (Bos indicus x Bos taurus) synchronized to fixed time embryo transfer

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Introduction

Previous research has demonstrated effectiveness of trace mineral supplementation with MULTIMIN 30 days prior to calving and breeding in increasing conception rates (1). The objective of this trial was to evaluate the effect of MULTIMIN supplementation (100 mg Zn, 100 mg Mn, 50 mg Cu and 25 mg Se) 17 days prior to fixed embryo transfer (FTET) on the survival rate of embryos in recipient cows.

Material and Methods

The experiment was performed from November (2007) to July (2008) on 3 farms in Brazil (the number of animals in each farm according to control or Multimin groups were respectively 40 and 40 in Uberaba, 45 and 47 in Uberlândia and 69 and 71 in São João da Boa Vista). All heifers received a free choice mineral supplement ad libitum. Crossbred heifers (n = 312), with body condition scores between 2.75 and 3.50 (1 to 5 scale) were randomly assigned to one of two treatments. The experimental groups were: a) Control - animals that did not receive any kind of injectable mineral supplementation and b) Multimin – animals that were treated with 5 ml Multimin subcutaneous at the beginning of FTET protocol. All heifers were treated with the following protocol: estradiol ester administration i.m. with the intravaginal-progesterone releasing insert or a Norgestomet ear implant. Eight or nine days later the implant/device was removed and eCG, PGF and estradiol cypionate were administered i.m. The FTET was performed on Day 17. Pregnancy and embryonic losses rates were evaluated by ultrasonography exam at 30 and 60 days after FTET, respectively. The data were analyzed using the GLIMMIX procedures of SAS. The explanatory variables utilized in the model were embryo quality, body condition score and location. The significant level to reject H0 (nullity hypothesis) was 5%.

Results and Discussion

No effect of injectable trace mineral supplementation was detected. However, a numerical increase of 10% on conception rate was observed for treated group at 30 and 60 days after FTET (Table 1). There was no statistical difference on variables included in the model ($P > 0.05$). There was no interaction between farms and treatments. Some of the animals that were able to embryo transfer (Group Control, n = 124; Group Multimin, n = 127) did not receive an embryo (Group Control, n=10; Group Multimin, n = 18) due to the number of available embryos to ET. Further research with increased numbers of animals is needed to evaluate the effect of injectable trace minerals MULTIMIN 17 days prior to ET on the reproductive responses to hormonal treatments.

Table 1. Effect of injectable mineral (Multimin) on response of FTET in crossbred heifers.

Rates (%)	Control	Multimin	P
Transferable/synchronized	80.5 (124/154)	80.4 (127/158)	0.99
Conception 30 days after FTET	39.5 (45/114)	49.5 (54/109)	0.14
Conception 60 days after FTET	35.1 (40/114)	44.9 (49/109)	0.13
Pregnancy losses	11.1 (5/45)	9.25 (5/54)	0.41

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Plasmatic progesterone concentration and pregnancy rate of bovine females synchronized to FTAI with Sincrogest® (intravaginal progesterone device)

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Introduction

The effect of the progesterone (P4) on bovine estrous cycle synchronization has been related for decades. In those previous studies, the animals were treated with daily progesterone injection. This method resulted in high heat synchronization. However, this practice has showed low fertility and low practicality (1). Nowadays, there are efficient products of controlled progesterone releasing used to synchronize the ovulation. The aims of these devices are to maintain higher plasmatic progesterone concentration (2). The objectives of these experiments were to evaluate the plasmatic P4 concentration and the pregnancy rate on FTAI in animals treated with a new intravaginal P4 device.

Material and Methods

On Experiment 1, 14 ovariectomized heifers were randomly allocated between the groups according to: number of use of P4 devices (1st, 2nd and 3rd use) and different P4 devices (Sincrogest®, Ouro Fino, Brazil or Primer®, Tecnopec, Brazil). An intravaginal P4 device was inserted in all animals and it was maintained until day 8 (192 hours after insertion). Blood samples were collected to determine the plasmatic P4 concentration at 0, 24, 48, 72, 96, 120, 144, 168, 192 (time of device withdrawal), 195, 204 and 216 hours after the device insertion. On Experiment 2, 155 crossbred cows were submitted to traditional protocol used to synchronize the ovulation for FTAI but using the different P4 devices [Sincrogest (n = 80) or Primer (n = 75)]. The statistical analysis was accomplished by proc GLM and GLIMMIX of the Statistical Analyses System.

Results and Discussion

On Experiment 1, the plasmatic P4 concentrations 24 hours after device insert reached 2ng/mL and showed similar patterns of releasing and maintenance of P4 during 196 hours (device period, Fig. 1, 2 and 3) in both groups (new and previously used devices). It has been verified similar pregnancy rates between the treatments [58.8% (47/80) for Sincrogest® vs. 52.0% (39/75) Primer®]. In conclusion, Sincrogest® kept plasmatic P4 concentrations over than 1ng/mL during the treatment with the devices (1st 2nd and 3rd use) and it can be used in synchronization of ovulation protocols for timed artificial insemination in bovine.

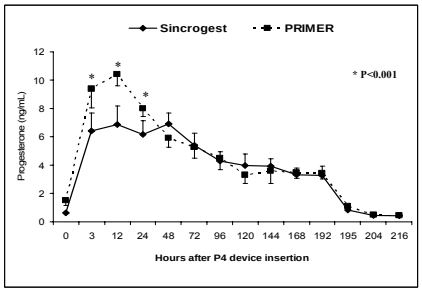

Figure 1. Plasmatic progesterone concentration of ovariectomized heifers treated with a new P4 intravaginal device.

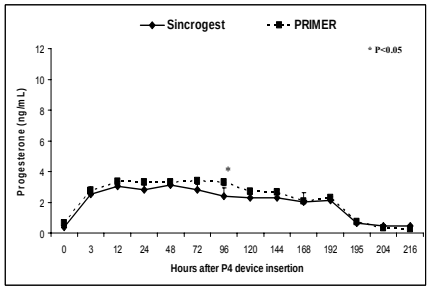

Figure 2. Plasmatic progesterone concentration of ovariectomized heifers treated with a previously used for 8 days P4 intravaginal devices.

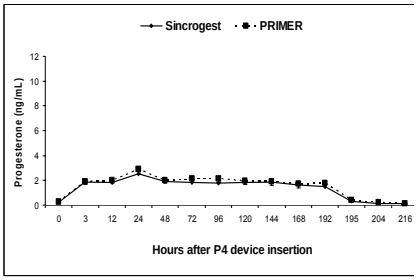

Figure 3. Plasmatic progesterone concentration of ovariectomized heifers treated with a previously used for 16 days P4 intravaginal devices.

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Acknowledgments: Ouro Fino Saúde Animal and Fazenda Bosque Belo, Aquidauana/MS.

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Effect of different ovulation inductors on pregnancy rates of Nelore (*Bos indicus*) heifers synchronized to fixed-time artificial insemination

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Introduction

The synchronization of ovulation protocols has obtained satisfactory results in *Bos indicus* cows, allowing the fixed-time artificial insemination (1). However, the protocols developed to lactating cows are presenting lower results when used in *Bos indicus* heifers. So, the objective of this experiment was to evaluate the pregnancy rate and the diameter of dominant follicle at artificial insemination when Estradiol benzoate (EB), Estradiol Cypionate (EC) or GnRH was used as ovulation inductor in synchronization of ovulation protocols that allow fixed-time artificial insemination (FTAI) in Nelore (*Bos indicus*) heifers.

Material and Methods

It was used 619 cyclic (presence of CL) Nelore heifers with body condition score (BCS) of 3.02±0.34 (1 to 5 scales) and ages 22 to 28 months that was assigned in a factorial design. At day 0 (AM), all animals received 2mg of EB (Estrogin®, Farmavet, Brazil) and a Norgestomet ear implant [randomly distributed between new (n = 305) or previously used for 9 days (n = 314); Crestar®, Intervet, Netherlands]. On Day 8, the heifers were allocated in one of three groups, considering the BCS and the type of implant (new or used). At this day, the implant was removed, 150µg of d-Cloprostenol (Preloban®, Intervet, Netherlands) and 300IU of eCG (Folligon®, Intervet, Netherlands) were administered in all animals. The heifers of CE group (n = 205) received 0.5mg of EC (ECP®, Pfizer Saúde Animal, Brazil) at that moment, the animals of BE group (n = 218) received 1.0 mg of EB 24 hours later and the heifers of GnRH group (n = 195) were treated with 100µg of gonadorelin (Fertagyl, Intervet, Netherlands) at FTAI that was performed 54 hours after implant withdrawal in all animals. Ultrasonographic exams were performed to verify the diameter of largest follicle in the ovaries at FTAI moment. The pregnancy diagnosis was performed 30 days after FTAI by ultrasonography. The statistical analysis was accomplished by proc GLM and GLIMMIX of the Statistical Analyses System (SAS).

Results and Discussion

There was no interaction between the type of implant and ovulation inductor in any responses variables. The results for CE, BE and GnRH or new and used norgestomet ear implant are presented in table 1.

Table 1. Pregnancy rate and diameter of bigger follicle at FTAI according to ovulation inductor or type of norgestomet ear implant.

Treatments	Ø of > follicle at FTAI (mm)	Pregnancy rate (%)
Estradiol benzoate	8.28 ± 0.03^{b}	40.4 (88/218)
Estradiol cypionate	8.09 ± 0.03^{b}	48.3 (99/205)
GnRH	9.39 ± 0.03^{a}	48.7 (95/195)
New ear implant	9.20 ± 0.02^{a}	46.6 (142/305)
Used ear implant	7.90 ± 0.03^{b}	44.3 (139/314)

Different letters at same column show statistical difference ($p<0.05$)

Conclusion

The use of GnRH allowed a higher diameter of dominant follicle at FTAI compared to EB and EC. However, this effect did not affect the pregnancy rate. A similar effect was observed on new or used norgestomet ear implant. The new implant allowed higher follicular diameter but it did not affect the pregnancy rate.

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Progesterone plasma profile after intramuscular progesterone injection in non-cycling mare

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Introduction

Progesterone (P4) is the main hormone responsible to maintain the pregnancy. Some studies have demonstrated that it is possible to maintain the pregnancy in ovariectomized mares using exogenous progesterone (1,2). Thus, the objective of this experiment was to evaluate the administration of injectable progesterone (Sincrogest injetável) in the P4 plasma concentration and pregnancy rate after embryo transfer (ET) in non-cycling mares.

Materials and Methods

On Experiment 1, twenty non-cycling mares were randomly allocated in one of two treatment groups: The mares of P4OF-A Group (n = 10), received a single injection of 3.5 mg/kg i.m. of injectable progesterone in oil vehicle type A (concentration of 150mg/mL) and the mares of P4OF-B Group received a single injection of 3.5mg/kg i.m. of injectable progesterone formulated using other oil vehicle (type B; same concentration than type A). Blood samples were collected by jugular venipuncture to determine the progesterone plasma concentration (radioimmunoassay) at 0, 24, 48, 72, 96, 120, 144 and 168 hours. At Experiment 2, 49 crossbreed mares received 6mg of Estradiol benzoate, (Sincrodiol, Ouro Fino, Brazil) in two consecutive days [D0 (random day that was chosen as the beginning of the experiment) and D1]. On D3, the animals that showed uterine edema greater than 3 (1 to 5 scale) received a single injection of 3.5mg/kg i.m. of injectable P4 in oil vehicle type A (Sincrogest Injetável, Ouro Fino, Brazil). This formula was chosen due to the facts of the smaller cost and local reaction. The females that received injectable P4 were embryo transferred between D7 and D11. After the embryo transfer, the mares were supplemented with weekly of single injections of progesterone until day 120 of pregnancy. The mares that were diagnosed as pregnant at day 15 were monthly monitored until 120 days of pregnancy.

Results and Discussion

At Experiment 1, the progesterone sources showed similar P4 releasing profile that was kept over 3 ng/mL per 7 days (Fig. 1), moreover, there was no difference in the maintenance of the plasmatic progesterone concentrations at the end of the experimental period. According to Daels (3), concentrations over than 2 ng/mL are sufficient to maintain the pregnancy in mare. The results of Experiment 2 indicate that the P4 used is efficient to maintain the pregnancy in mares until 120 days (Table 1). Thus, it can be concluded that Sincrogest injectable once a week is an alternative to facilitate the management in equine recipients in ET, allowing the use of non-cycling mares.

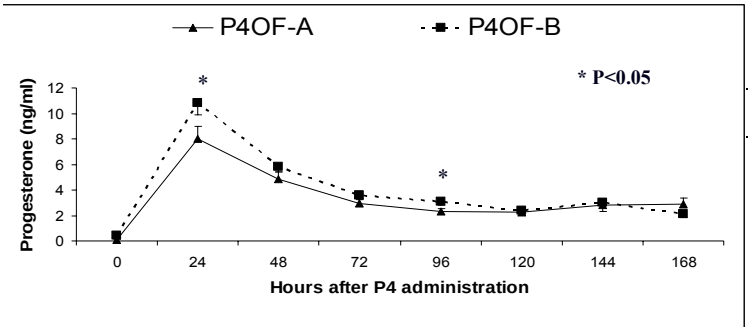

Figure 1. Progesterone plasma concentration after the injectable P4 administration in mares.

Table 1. Effect of the treatment with injectable progesterone on the pregnancy rate of non-cycling mares submitted to embryo transfer.

Rate (%)	
Conception 15 days after ET	73.5 (36/49)
Conception 120 days after ET	65.3 (36/49)
Pregnancy losses (120 days)	11.1 (4/49)

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Acknowledgments: Ouro Fino Saúde Animal, Haras Bandeirantes and Central de Reprodução Eqüina Dutra.

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Pregnancy by artificial insemination with cooled epididymal spermatozoa obtained from post–death stallion - case report

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Introduction
The recovery of spermatozoa from the epididymis cauda may be an important tool in equine reproduction because it makes possible the recovery of viable cells after the death of valuable stallions. During sperm maturation inside the epididymis, the plasmatic membrane is biologically modified, which will provide motility and capacity to fertilize the oocytes (1). The purpose of this case was to report the viability of the spermatozoa recovered from the epididymis cauda after stallion death.

Materials and Methods
Epididymal was obtained from one stallion (Paint Horse), 14 years old that died from colic syndrome. One hour after its death, it was recovery one of the testicles, the other was atrophied after a previous trauma. The testicle was maintained in a 0.9% saline solution in an ice box for 19 hours. The recovery of the spermatozoa was accomplished by compressing the epididymis cauda and part of the deferent duct with help of an anatomic nipper on a Petri dish containing the extender Botu-Turbo® (Biotech Ltda, Botucatu, Brazil). The sperm samples were evaluated for motility, vigor, sperm concentration, percentage of viable cells and morphology. The semen was diluted (1:2) and fractionated in two doses with 2.1 x 10^9 total sperm each. The semen doses were refrigerated at 5ºC for 4 hours and two mares (Quarter horse) were artificial inseminated.

Results and Discussion
The average motility was 80%, vigor 4, concentration was 4.2 x 10^9 total sperm, the percentage of alive spermatozoa was 85% and of normal spermatic cells was 60%. The most frequent spermatic alterations were proximal cytoplasmic droplet (29%) and strongly bended tail (8%); probably because the sperm was from epididyminal cauda. Through ultrasound examination, it was found that mares had corpus luteum (ovulation) 14 hours after insemination. Twenty-three days after artificial insemination the pregnancy was confirmed by ultrasound diagnostic of the mares. The results showed that the process of spermatozoa recovery and refrigeration obtained from equine epididymis cauda was efficient for the maintenance of spermatic in vivo fertility. This report confirms results of previous studies in our laboratory (2), which means that the process of spermatozoa recovery and refrigeration obtained from the epididymis cauda was efficient for the maintenance of spermatic quality after 24 hours at 4 ºC. The time between the death of the animal and the recovery of the testicle is essential to the preservation of genetic material.

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Morphological analysis of pig preantral follicles in ovarian tissue submitted to cold storage followed by cryopreservation

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Introduction
Expecting to preserve genetic material from animals threatened of extinction and with high genetic value, researchs have been conducted to develop efficient methods to conserve germ cells. In pigs, female gametes have high sensibility to cold, and cryopreservation of oocytes from antral follicles presents poor results (1). Cryopreservation of preantral follicles represents an alternative to preserve pig female germ cells. In fact, a great number of morphologically normal preantral follicles (MNF) are present in cold stored pig ovaries (2). Cryopreservation of pig ovarian tissue has also been tested, and both ethylene glycol (EG) and DMSO allowed the survival of a good percentage of MNF (3). The objective of this work was to test the effect of cooling followed by freezing of ovarian tissue on the morphology of pig preantral follicles.

Materials and Methods
Ovaries from 8 gilts were collected at a local slaughterhouse. From each ovary 4 slim slices of cortex (~ 0.5 x 2 x 5 mm) were cut, and 1 slice was immediately fixed in Carnoy fixative (Control Group). Samples were placed in PBS at 4ºC and kept at 4°C for 18 hours. After that, one sample was fixed (Chilling Group). The other two samples were cryopreserved in 1.5M of EG with 0.4% sucrose. After 5 days, samples were thawed and cryoprotectant was removed. Then, one of the samples was fixed (Cryo Group) and the other sample was placed in *in vitro* culture (3) for 2 h and then fixed (Cryo-IVC Group). For histological analysis samples were dehydrated in alcohol, clarified with xylene and imbedded in paraffin wax. Sections (5 μm) were stained with HE and analyzed by light microscopy. The percentage of MNF was compared among treatments by ANOVA and Tukey test.

Results and Discussion
The percentages of normal follicles in Control, Chilling, Cryo and Cryo-IVC treatments were respectively: 83 ± 9, 81 ± 9, 83 ± 10 and 73 ± 11. No statistical difference was observed among treatments ($P > 0.05$). Examples of pig preantral follicles from the control and Cryo treatments are shown in Figure 1. These results agree with those found for the chilling (2) and cryopreservation (3) of pig ovaries. In conclusion, it is possible to cryopreserve pig ovarian tissue after a period of low temperature storage with high percentages of morphologically normal preantral follicles. Deeper studies should be done, including the analysis by transmission electron microscopy.

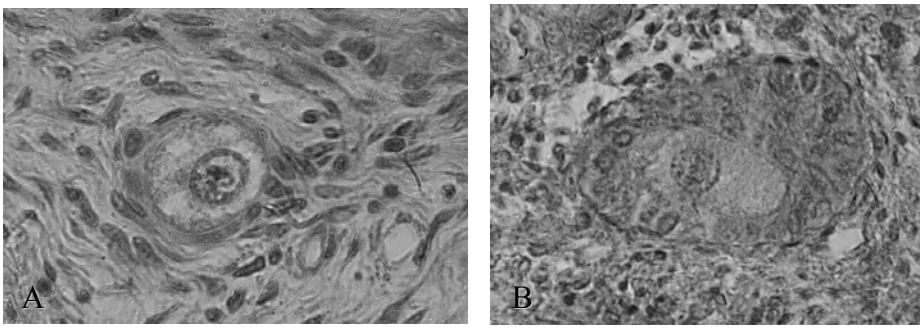

Figure 1. Morphologically normal pig preantral follicles from control tissue (A), and after chilling and cryopreservation of the ovarian tissue (B).

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Effect of dilution of homologous seminal plasma on the viability of frozen-thawed dog spermatozoa

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Introduction

The prostatic fluid does not seem to be a requirement for fertility in dogs since bitches conceive and deliver healthy pups after being inseminated with spermatozoa from the cauda epididymis. However, Nothling and Volkmann (1993) reported that the prostatic fluid improves the fertility of thawed dog semen. More pups were produced when bitches were inseminated using frozen-thawed semen diluted with seminal plasma. The aim of this study was to evaluate the effect of dilution of homologous seminal plasma on kinetic parameters of frozen-thawed dog spermatozoa.

Materials and Methods

Eight adult dogs were used in this study. Semen collection was performed via digital stimulation in the presence of an estrous bitch. The ejaculate was analyzed, centrifuged and seminal plasma was discarded. The *pellet* was resuspended in cryopreservation extender (Tris/citric acid/egg yolk/glycerol/OEP/amikacin) in a single step. After thawing, semen was extended in Tris without glycerol (*control group*), or in homologous seminal plasma (*experimental group*). Sperm motility was evaluated immediately after thawing and 30 minutes later using a computer assisted semen analysis (CASA). Membrane integrity was assessed using fluorescent probes after thawing and 30 minutes later.Homologous seminal plasma used to dilute the thawed semen was obtained from a dog that was trained for semen collection. After collection, the ejaculate was centrifuged, seminal plasma was separated, and then divided into aliquots and stored frozen until analysis. Statistic differences between groups were analyzed through ANOVA.

Results and Discussion

Table 1. Results of motility parameters evaluation by CASA and sperm membrane integrity. Botucatu, 2008.

	T0			T30		
Variables	TRIS	Plasma	p	TRIS	Plasma	p
NCT	556,6	853,7	0,30	470,10	467,1	0,90
MT	49,5	41,5	0,50	33,70	34,6	0,90
MP	37,0	31,2	0,50	23,30	27,3	0,60
VAP*	79,0	98,1	0,008	80,50	100,2	0,03
VSL*	71,1	85,6	0,02	70,70	89,2	0,04
VCL	109,4	144,8	0,01	114,50	148,3	0,05
ALH	5,0	6,4	0,08	6,30	6,6	0,70
BCF*	21,5	25,1	0,002	22,40	21,0	0,40
STR	89,2	85,5	0,05	87,20	87,5	0,90
AREA*	4,8	5,9	0,007	4,60	5,3	0,09
M*	9,7	5,8	0,04	8,00	4,5	0,12
IM (%)	56,2	63,0	0,30	45,0	44,1	0,80

Significant difference was found among different extenders and among moments for the following sperm velocity parameters: average path velocity (VAP) and straight line velocity (VSL). It was also observed a statistically significant difference between TRIS and seminal plasma for beat cross frequency (BCF), area and mean velocity (M) immediately after thawing. No differences were observed 30 minutes after thawing. In conclusion, dilution of frozen-thawed dog semen with homologous seminal plasma favors some sperm movement parameters, and is thus indicated for artificial insemination procedures.

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Financial support:CNPq

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Cryopreservation of preantral follicles in queens ovarian tissue (Preliminary results)

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Introduction

At birth, mammalian ovaries are endowed with hundreds of thousands of preantral follicles. A few studies have been done with cryopreservation of these follicles in queens (1), however an efficient method for long-term conservation of immature oocytes in preantral follicles was not yet established. Moreover, a vitrification method was not tested in queen ovarian tissue as well. The aim of this study was to cryopreserve queen preantral follicles in ovarian tissue by slow freezing and vitrification.

Material and Methods

Ovaries from 5 queens were collected and sectioned. One sample of ovarian cortex was immediately fixed (control). Other samples were submitted to the slow freezing (SF) or vitrification (V) treatment. For the SF, samples were equilibrated in DMSO 1.5M + sucrose 0.4% in PBS for 20 min at 10°C. Thereafter, samples were transferred to a cryomachine, cooled at 1ºC/min to -7ºC, manually seeded, cooled at 0.3ºC/min to -30ºC, and plunged into liquid nitrogen. In the V method the ovarian samples were equilibrated in DMSO 1M for 5 min at room temperature, transferred to DMSO 2M + Propanediol 3M for 5 min. After that period, the samples were vitrified in a solid surface and plunged into liquid nitrogen. After 1 week, fragments from the SF were thawed and the cryoprotectant was removed. For V protocol, samples were warmed and the cryoprotectant removed as well. After that, samples were processed for classical histology and follicles were classified as morphologically normal (MNF) or degenerated. Additionally, samples were processed for routine ultrastructural analysis. Data from histology were compared by ANOVA and Fisher's PLSD test.

Results and Discussion

On the control group 89.1 ± 4.8 of the follicles were classified as MNF. The percentage of MNF was 60.6 ± 9.5 and 15.7 ± 5.7 for SF and V treatments, respectively, both significantly different from the control ($P < 0.05$). Moreover, the percentage of MNF in V was lower than in SF treatment ($P < 0.05$) (Fig. 1A). For ultrastructural analyses, the cryopreserved groups demonstrated some cryoinjuries in the oocytes. For SF, the most common alterations were disorganization of oocytes cytoplasm, which presented translucent mitochondria, few endoplasmatic reticulum cisternae and a higher number of vesicles (Fig. 1B). The ultrastructure of V follicles showed much more damages. In some follicles, the oocyte appeared disconnected from the granulosa cells (Fig. 1C), and the ooplasm was full of vesicles. In this work the V method demonstrated to be a more damaging protocol than the SF, however the SF group demonstrated some damages in the ultrastructure level as well. We can conclude by this report that both methods bring cryoinjuries to the oocyte, especially vitrification, but more studies must be held in order to elucidate better cryopreservation protocols helping not only the preservation of cats but especially of endangered wild cats.

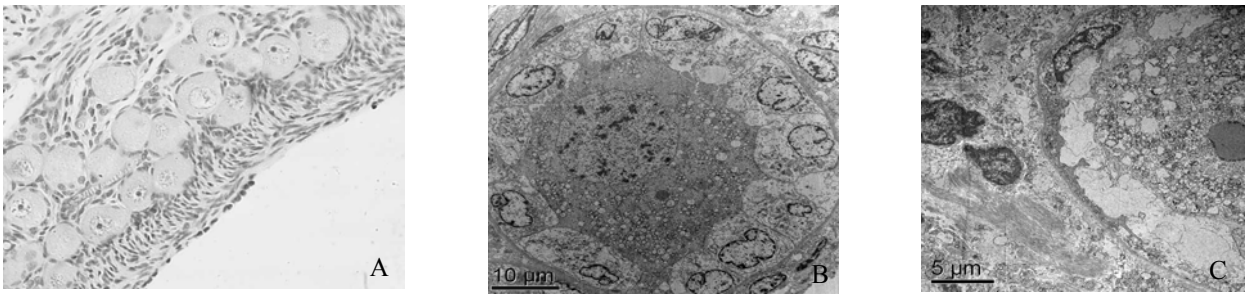

Figure 1. A. A group of morphologically normal preantral follicles. B. Preantral follicle after SF cryopreservation. C. Preantral follicle after vitrification showing a vacuolated oocyte detached from granulosa cells.

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Delivering cholesterol or cholesteryl conjugates loaded cyclodextrin on stallion sperm cryosurvival

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Introduction

Cryopreservation induces partially irreversible damage to sperm that result in reduced fertility for frozen sperm from many stallions compared to fresh or cooled ones. Previous studies indicate that the cholesterol/phospholipid ratio of the plasma membrane is a major determinant in plasma membrane fluidity and stability during cryopreservation. Techniques to increase sperm membrane cholesterol levels have increased cryosurvival rates of stallion sperm. To increase membrane fluidity at low temperatures, cholesterol may be added to the sperm membrane using cyclodextrins. The objective of this study was to compare the effect of adding different cholesteryl conjugates loaded cyclodextrins in stallion sperm prior to cryopreservation to optimize its cryosurvival.

Materials and Methods

Methyl-β-cyclodextrin was loaded with cholesteryl conjugates as described by (1). Ejaculates (n = 12) from 5 stallions were collected using artificial vagina and immediately following collection, the sperm concentrations and the percentages of total and progressively motile spermatozoa were determined using a densimeter and CASA, respectively. Each ejaculate was diluted 1:1 (v/v) in STALP and centrifuged in 50 ml conical tubes at 400 g for 7 min. The sperm pellets were suspended to $120x10^6$ sperm/ml with STALP and subdivided into 14 treatments: No additive (control); 1.5 mg cholesterol loaded cyclodextrin (CLC)/$120x10^6$ sperm (positive control); 1.5 or 3.0 or 4.5 mg of cyclodextrin pre-loaded with cholesteryl conjugated (heptanoate, palmitate, pelargonate or stearate). All portions were held at 22 ºC for 15 min. After, the semen was diluted 1:5 (v/v) with lactose-egg yolk diluent and cooled to 5 ºC over a 2 h period. Loaded into 0.25 ml straws, frozen in liquid nitrogen vapor for 10 min, and then plunged into liquid nitrogen until further use. Straws were thawed in a 37 ºC water bath for 30 sec, and a subsample analyzed by CASA for total and progressive motility. The contents of each straw were diluted 1:4 (v:v) in lactose-egg yolk diluent to final spermatozoa concentration of $20x10^6$ cells/ml and each sample maintained at room temperature for approximately 10 min before analysis. Treatment differences for sperm motility was determined using ANOVA and SNK at $P < 0.05$.

Results and Discussion

After thawing the total and progressive sperm motility was higher in stallion sperm containing 1.5 mg CLC (52% and 31%, respectively) compared to sperm treated with other cholesteryl (34.6-46% and 15-27%, respectively) and non-treated (44.2% and 22.8%, respectively, $P < 0.05$). Stallion sperm are damaged during cryopreservation due to ice crystal formation and to plasma membrane damage when it undergoes a phase transition from liquid to crystalline/gel state (2). Increasing the plasma membrane cholesterol/phospholipid ratio reduces the temperature at which the membrane phase transition occurs and may eliminate it completely. This study confirms that treating stallion spermatozoa with CLC (1.5mg CLC/120 million spermatozoa) prior to cryopreservation increases the percentages of motile spermatozoa after thawing (3). The data reported in this study show the positive results of CLC addition to stallion sperm by using in vitro laboratory assays including increasing the percentages of motile. Control samples and sperm treated with cholesteryl-heptanoate, -palmitate, or -stearate loaded cyclodextrin exhibited similar percentages of motile sperm after freezing ($P > 0.05$). Therefore, adding cholesterol to stallion sperm membranes improved cell cryosurvival, whereas treatments with cyclodextrins pre-loaded with other cholesterol-like molecules did not.

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Cryopreservation of testicular tissue and spermatogonial cells from Nile tilapia (*Oreochromis niloticus*)

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Introduction

In a previous study we have demonstrated that spermatogonial cells can be successfully transplanted directly into tilapia testis (Lacerda et al 2006), and these are capable to form fully differentiated spermatozoa with donor characteristics (unpublished data). Cryopreservation of gonadal tissue and germ cells is a very useful technique that could be utilized for instance to preserve the genetic diversity of threatened or endangered wild fish populations. In the present study, we aimed to investigate the possibility of cryopreserving spermatogonial cells and testis fragments from sexually mature tilapias, utilizing two different protocols, prior to the germ cells could be utilized for transplantation.

Materials and Methods

Testicular fragments: Sexually mature tilapia testicular tissue fragments were placed in 1.5mL cryotubes containing cryoprotectant medium (5% glucose, 10% hen egg yolk, 10% DMSO, and 75% distilled water) or only DMEM. Subsequently, the samples were cooled in a dry-shipper for 24 h, transferred into liquid nitrogen (-196˚C). The samples were thawed in water bath at 25˚C, washed with DMEM and processed for light microscopy. Freshly collected tissue fragments were evaluated as a control under light microscopic analysis.

Cell Suspensions: Testes from sexually mature tilapias were digested, and a spermatogonia enriched cell suspension was obtained by Percoll density gradient centrifugation. Aliquots of 500µL of cell suspension (10^5 cells/mL) were carefully added to an equal of volume of freezing medium (10% fetal bovine serum, DMEM-C, DMSO 10%) or only DMEM, and distributed in 1.5 mL freezing vials. Samples were frozen at uncontrolled and controlled cooling rate (-1˚C/min.) up to -80^{o}C and then transferred into liquid nitrogen. For thawing, the tubes were plunged in a water bath for 1-2 min at 25˚C and the cryoprotective agent was removed. Cell viability was assessed by trypan blue staining. Subsequently, all samples were fixed and processed for histological analyses.

Results and Conclusion

Testicular fragments: Although some areas showed detachment of spermatogenic cysts from de basement membrane comparable to control, tissue cryoprotected with DMSO displayed well-preserved cells. Testis fragments frozen without any cryoprotectants showed disrupted stroma and parenchyma, as indicated by condensed chromatin in the nuclei, clearly altered cytoplasm and disrupted cell-cell contact.

Cell Suspensions: Cell viability assessed by trypan blue dye-exclusion in uncontrolled and controlled freezing protocols is shown in Fig. 1.

As it is shown, DMSO as a cryoprotectant improved the general preservation of tilapia testicular fragments as well as the viability of isolated spermatogonia. These promissing preliminary findings obtained herein suggest that cryopreservation might be a potential tool for future studies involving germ cells transplantation and testis graft in fish.

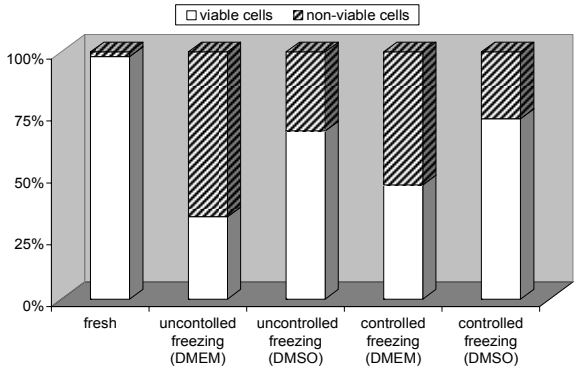

Figure 1. Survival rates of fresh and frozen/thawed selected tilapia spermatogonial cells.

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Construction of a lentiviral vector containing interfering microRNA for the generation of livestock with myostatin gene knockdown

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Introduction

Production of transgenic livestock was demonstrated to be feasible over three decades ago. It became apparent almost immediately that the method used to produce the transgenic livestock had substantial limitations that would obstruct its use both for research and commercial applications (1). The advantage of using lentiviruses as vectors for delivering transgenes is that lentiviral genome can be actively transported into the nucleus, allowing it to deliver transgenes to non-dividing cell types (2). The aim of this study was to propose the use of lentiviral based vector containing interfering microRNA targeting myostatin for the transduction of bovine zygotes.

Materials and Methods

This experiment used the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (Invitrogen Corporation) (3). To generate the Double-Stranded oligo (ds oligo), 200 µM of Top strand-TGCTGTCAGGATTTGCACA AACACTGGTTTTGGCCACTGACTGACCAGTGTTTGCAAATCCTGA and of bottom strand-CCTGTC AGGATTTGCAAACACTGGTCAGTCAGTGGCCAAAACCAGTGTTTGTGCAAATCCTGAC were annealed on a heat block at 95ºC for 4 minutes. The ds oligo was cloned into the pcDNA™6.2-GW-EmGFP-miR vector which was then used to transform One Shot® TOP10 competent *E. Coli*. The analysis of positive clones was performed by PCR (polymerase chain reaction) selecting 10 colonies with the use of miR forward primer-GGCATGGACGAGCTGTACAA and reverse primer-ACAAAGTGGTTGATCTAGAG. Positive colonies' plasmid DNA was isolated using the Mini Plasmid Purification Kit (PureLink™, Invitrogen Corporation). To evaluate the insert orientation the vector was digested with MscI, which cuts once within the miRNA loop and once elsewhere in the backbone. The same procedure was performed with the control sequence miR-lacZ.

Results and Discussion

The annealing step was successful (Figure 1A). In the PCR two colonies out of 10 were positive for miR_Myost and one out of 4 control colonies were positive for miR_lacZ (Figure 1B). Plasmid DNA isolation had a good performance reaching 70 ng/µL in average and the recombinant vectors presented the correct orientation as both fragments (4333 e 1422 bp) were generated after MscI digestion. This vector will be used for recombination in the intermediate pDONR™ vector.

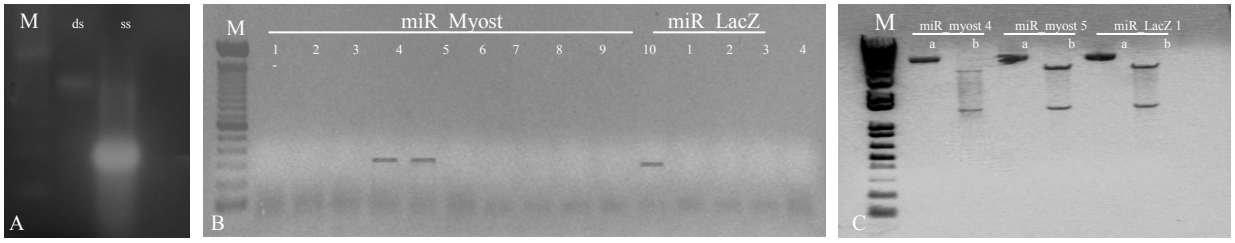

Figure 1. (A) Annealing of oligonucleotides, (ds) Double stranded oligo, (ss) Single stranded oligos; (B) Colonies characterization by PCR, 274 bp fragments exhibits recombinants (miR_Miost samples 4 and 5, miR_LacZ sample 1), (-) negative control; (C) MscI digestion assay, (a) pDNA_6.2/gw_miR_Miost or LacZ circular 5755 bp, (b) 4333 and 1422 bp fragments. (M) 1 kb DNA ladder.

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Use of the powdered coconut water for the conservation of canine preantral ovarian follicles

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Introduction

In dogs, studies concerning the conservation of preantral ovarian follicles are limited, but the use of saline solutions as conservation media for ovary fragments has been demonstrated (1). In goats, the coconut water based solution was demonstrated as an efficient conservation medium for ovary structures *in situ* (2). The present study aimed to determine the effect of the powdered coconut water (PCW®) as a conservation medium for canine preantral ovarian follicles *in situ*.

Material e Methods

The ovarian pairs from bitches (n = 6) were collected and divided in seven fragments. A first fragment from each pair was immediately destined to classic histological evaluation; the others fragments were conditioned in plastic tubes containing phosphate buffered saline (PBS – control group) or PCW®, and stored in 5L isothermal boxes (15 x 21 x 17 cm), containing 3L biological ice. The boxes were sealed and the sensor of a digital thermometer was put into it. The boxes were open after 12, 24 and 36 h and the ovarian fragments were submitted to the histological evaluation. The percentage of intact and degenerate preantral ovarian follicles was evaluated. The internal temperature of the boxes and the pH of the media were checked in each evaluation. Six replicates were conducted for each treatment. The results are showed in average and standard deviation and the comparisons among the media were performed by Mann-Whitney test ($P < 0.05$). The effect of the conservation time on the follicular integrity was analyzed by Student's t test ($P < 0.05$).

Results and Discussion

The results are in Table 1. No differences were verified between the conservation media ($P > 0.05$). A significant reduction of the follicular integrity started from 24 h for both media ($P < 0.05$), however 50% of the ovarian follicles were intact even at 36 h, making possible their use in cultivation or cryopreservative procedures. We conclude that the powdered coconut water (PCW®) can be used as an alternative conservation medium for canine preantral ovarian follicles under cooling in a short period of up to 36 h, but the accomplishment of more specific studies for evaluation of its effect on the oocyte viability is suggested.

Table 1. Histological integrity of canine preantral follicle conserved *in situ* in phosphate buffer saline (PBS) or powdered coconut water (PCW®) for up 36 h into isothermal boxes.

Time of conservation	Follicular integrity (%)	
	PBS	PCW®
0 h	80.3 ± 11.5^{a}	80.3 ± 11.5^{a}
12 h	$73.8 \pm 6.6^{a,b}$	$79.3 \pm 6.9^{a,b}$
24 h	59.0 ± 20.6^{b}	71.7 ± 8.0^{b}
36 h	61.5 ± 10.3^{b}	53.8 ± 12.3^{b}

No differences were verified between media ($P > 0.05$);
^{a,b}Values followed by different low case letter differ between rows ($P < 0.05$).

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Postzygotic isolation study between different cytotypes of *M. americana* through hybrid female reproductive analysis

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Introduction

The *Mazama americana* species is the most controversial neotropical deer from a taxonomic, molecular and cytogenetic point of view. The species is distributed throughout Brazil and is genetically characterized by a distinctive cytotype, which is due to chromosome polymorphism with some geographic coherence. This study evaluated postzygotic isolation in reproduction isolation via analysing the fertility of karyotype outbreed females'.

Material and Methods

We produced three hybrids females by crossing the following different karyotypes: 2n = 53 x 2n = 43 (female 1); 2n = 45 x 2n = 52 (female 2); 2n = 45 x 2n = 50 (female 3). To evaluate the fertility of these females we conducted the following evaluations: i) monitoring the estrous cycle by measuring fecal progestagens from 6th to 18th months of age; ii) analysis of the ovary function by ovary histology and subsequent observation of the presence or absence of different follicle stages; iii) evaluation of the oocyte fertility through embryo *in vitro* production (IVP) after follicular aspiration and slicing technique (for follicular aspiration we used the superovulation treatment); iv) observation of pronuclei presence after performing the nuclear staining technique (Hoechst 33342).

Results and Discussion

The first female's (female 1) data differed from the other two females as follows: female 1 showed a delay in puberty (15th months of age); irregular estrous cycle (duration 21 to 56 days); absence of primordial and primary follicles; and a negative response to superovulation treatment. Furthermore, the IVP obtained just one oocyte (via slicing technique) from Female 1 that did not show any embryo cell division. In addition, the oocyte did not show a pronucleus on the nuclear staining, indicating that it was not fertile. The other two females (Females 2 and 3) showed the following results: puberty was observed around the same time (8th and 10th months of age); regular estrous cycle (duration 21 days); presence of primordial and pre-ovulated follicle and positive response to superovulation treatment. From Female 2 and 3, twenty-two oocytes were obtained via combination of oocyte aspiration and slicing technique for IVP. From these, 18 had an embryonic development and four did not show any embryonic cell division. At the nuclear staining we observed one embryo (from Female 3) with two pronuclei, and one embryo (from Female 2) with three pronuclei (polyspermy), indicating oocyte fertility of Females 2 and 3. Additionally, the positive response to superovulation treatment and the embryo production corroborates the results of reproduction capacity in Females 2 and 3. Female 1 results were the most distant karyotype crossing with 10 chromosomes of difference, while the other females had a chromosomal difference of 7 and 5, respectively. In conclusion, we believe that the infertility of Female 1 may be a result of the distance between parental chromosome numbers. In addition, the results from Females 2 and 3 showed normal ovarian function despite the chromosome differences, which may indicate a possible fertility. Further studies will be made to improve the results and to confirm if a real postzygotic isolation exists between the chromosomal variants of *M. americana*.

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Ovarian papilliferous carcinoma in cat: case report

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Introduction

Although uncommon, ovarian tumours have been described in all domestic species. Exceptionally in queens, ovarian tumours are extremely rare. In a retrospective study of 138 cases of urogenital tumours, 95% were seen in dogs, and only 5% in cats. Ovaries and bladder were the most affected sites. In another study that evaluated 22 cases of ovarian tumours in queens, one had an epithelial origin, seven had a germinative cell source, and 14 were developed from granulosa cells. In all domestic females, except the bitch, epithelial tumours are uncommon. Most of these tumours arise from the superficial epithelium of the ovary, or from the *rete ovarii*. Grossly, they are cystic with dense focal areas, and range in size from 7 to 10cm. Cysts are fluid filled with neoplastic cells content that can scatter from the primary carcinoma into the abdominal cavity forming metastasis. The aim of the present report was to describe clinical, surgical and histopathological aspects of a rare ovarian tumour in queens.

Material and Methods

A ten year-old, non-spayed, mixed-bread queen was presented; featuring weight loss, prostration, polydipsia, polyfagia, and fasting hyperglycemia (glycemia > 400mg/dL) for at least 6 months. The cat had been previously treated with Lantas glargine® insulin, with an improvement of clinical signs. Only two recent estrous cycles were reported during the queens' entire life. Physical examination revealed a round structure in the left hypogastric region of the abdomen. An enlarged mass measuring 8 cm appeared caudal to the left kidney on abdominal ultrasound. Laparotomy was performed for definitive diagnosis. During the procedure, a large, round, regular left ovary mass was found, and immediately the queen underwent ovariohysterectomy. No signs of abdominal metastasis were found. Histopathology revealed an ovarian papilliferous carcinoma.

Results and Discussion

Gross and microscopic findings were similar to those referred in the literature. Grossly, the tumour presented dark fluid-filled cystic and dense focal solid areas. Tubular and papillar pattern of the neoplastic cells, solid dense areas of moderated fibrovascular stroma were encountered at the histopathological evaluation. In addition, high magnification revealed cuboids to columnar tumour cells with a eosinophilic cytoplasma, and increased nucleus: cytoplasma ratios, such as ovate nucleus with decompacted chromatin, and distinguished nucleoli. Cells presented mild pleomorphism, high mitotic index (over five mitotic cells per field), and diffuse distribution of innumerous apoptotic cells. In bitches the susceptibility of the ovarian papilliferous carcinoma is due to epithelial sub-superficial structures, although in queens this type of tumour is extremely rare. High frequency of metastasis in the abdominal cavity is correlated to the poor prognosis of ovarian carcinomas, herein favorable prognosis was evidenced once no signs of metastasis were observed.

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Lecithin/Sphingomyelin ratio in amniotic fluid of canine neonates born under distinct obstetrical conditions

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Introduction

At birth, lung expansion during respiratory efforts decreases local vascular resistance, and consequently, enhances pulmonary blood flow and oxygenation (1). The main cause of the Respiratory Distress Syndrome in newborns (RDS) is the impaired production of pulmonary surfactant (2), as it is essential to decrease the superficial tension within the alveoli and prevents their collapse at the end of expiration. Surfactant synthesis by type II pneumocytes is associated with fetal lung maturation and it is of utmost importance for extra-uterine life (1). It may be indirectly documented in amniotic fluid and helps to predict neonatal lung immaturity. The objectives of the present study were to establish the L (lecithin) / S (sphingomielin) ratio in the amniotic fluid and to verify the influence of the obstetrical condition at birth on neonatal lung maturity.

Material and Methods

Forty-eight newborn puppies of different breeds and genders were grouped according to their obstetrical condition at term: Eutocia (n = 20); Dystocia (n = 8) and Cesarean section (n = 20). The amniotic fluid was collected asseptically from the intact amniotic sac protruded through the vaginal vault during labor or at hysterotomy. Samples were stored at –20^{o}C and the L/S ratio was obtained through. Results were tested using a repeated measure analysis of variance (ANOVA) and Newman-Keuls test with a significance level of 5% ($p < 0.05$).

Results and Discussion

The phospholipids lecithin and sphingomyelin concentrations, as well as the calculated L/S ratios are shown in table 1. Our results were similar to the L/S ratio proposed by Ducci, 2007 (3) for normal delivery puppies and otherwise higher than those obtained from equine and bovine newborns.

Table 1. Lecithin and sphingomyelin concentrations (µg/mL) and L/S ratio in Eutocia, Dystocia and C-section.

	EUTOCIA	DYSTOCIA	C-SECTION
Lecithin (µg/ml)	5.69 ± 12.54	5.27 ± 3.78	6.79 ± 7.03
Sphingomyelin (µg/ml)	0.88 ± 1.93	0.65 ± 0.56	1.12 ± 0.89
L/S Ratio	7.29 ± 3.55	8.54 ± 3.47	5.89 ± 4.37

no significant difference between groups ($p < 0.05$).

Despite no significant difference among groups, c-section puppies presented lower L/S ratio than the vaginal delivered ones. Respiratory anomalies are more frequently observed in c-section newborns (4). During normal labor, the rise in cathecolamines levels mediates a decrease in pulmonary fluid output, its higher absorption and stimulates surfactant secretion (5). The concentration of cathecolamines remains stable when c-section is performed prior to physiological onset of labor by the trigging rise in fetal cortisol concentration. Thus, final lung maturation can be impaired in puppies born chirurgically. In conclusion, canine neonates present L/S ratio superior than 5, regardless of the whelping condition, which denotes full lung maturation at term.

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Labor stress evaluation under distinct obstetric conditions in Holstein cattle

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Introduction

Calving is a physiological process which involves algesia, as well as maternal and fetal stress. The through course of labor leads to changes in hormonal levels of cortisol (1). However, the synthesis profile of this latest hormone by fetal adrenal suffers direct influence of different birth conditions (2). The aim of this study was to establish maternal and neonatal cortisol profile during different timepoints of calving at distinct obstetrical conditions.

Materials and Methods

Thirty Holstein cows and 30 Holstein calves were allocated into 3 groups according to the birth model as: eutocia (EUT; n = 10), dystocia (DYST; n = 10) and uterine inertia (OXY; n = 10). Dystocia was marked by the delay of the second stage of birth (superior to 2 hours) and severe obstetrical assistance. Uterine inertia was managed by ecbolic therapy (50 IU of oxytocin and 11.62 g of calcium gluconate) through slow infusion. Maternal blood samples were collected by coccigea vein punction at first stage of labor, intra-partum, immediately postpartum and 1 hour later. In order to assess neonatal cortisol concentration, blood sample was drawn by jugular punction at birth and after 60 minutes. Serum cortisol assay was performed through radioimmunoassay with the use of a Cortisol Coat-A-Count (DPC®) kit. Results were tested using a repeated measure analysis of variance (ANOVA) and Newman-Keuls test with a significance level of 5% ($p \leq 0.05$).

Results and Discussion

The second stage of labor had a full duration of 2 hours for group EUT, 3 hours and 30 minutes for group DYST and 3 hours and 50 minutes for group OXY. Cortisol concentrations are shown on table 1.

Table 1. Means $^{\pm}$ SD of peripartum serum cortisol concentration (ng/ml) in Holstein cows and calves at eutocia (EUT), dystocia (DYST) and uterine inertia (OXY).

		GROUP EUT	GROUP DYST	GROUP OXY
Cows	Prepartum	16.4 ± 3.4^{b}	15.4 ± 6.4^{b}	3.1 ± 8.4^{b}
	Intrapartum	29.6 ± 11.7^{a}	21.3 ± 6.7^{b}	19.9 ± 7.0^{ab}
	Postpartum	26.8 ± 9.8^{Bab}	42.4 ± 18.0^{Aa}	24.8 ± 6.7^{Ba}
	1h postpartum	33.9 ± 8.6^{a}	35.7 ± 6.2^{a}	25.8 ± 9.9^{a}
Calves	At birth	154.2 ± 47.4^{B}	213.0 ± 82.1^{Aa}	134.6 ± 33.8^{B}
	60 minutes	145.6 ± 28.4	160.2 ± 23.7^{b}	147.1 ± 53.4

^{A,B}in the same moment of evaluation indicates statistical difference ($p \leq 0.05$)
^{a,b}in the same group indicates statistical difference ($p \leq 0.05$)

All females presented higher cortisol levels 1 hour after calving compared to the prepartum evaluation. Cortisol concentration was statistical superior in the postpartum period in DYST group, possibly due to the sharp increase of stress in response of fetal extraction and also a longer period of fetal restraint in the pelvic canal. DYST calves showed superior cortisol levels immediately after birth as a consequence of the stress mediated by the obstetrical assistance. On the other hand, neonatal stress level of OXY calves at birth was similar to EUT group. In conclusion, dystocia followed by manual traction induces an increase on maternal and neonatal cortisol release immediately after birth, being considered a more stressful condition.

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Induction of postpartum ovarian activity in anestrous Indubrasil (*Bos taurus indicus*) cows

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Introduction

Indubrasil (Zebu) breed was developed in Brazil at the beginning of the 20th century from the three main Indian breeds (Nellore, Guzerat e Gyr). Its advantage use is to produce crossbred with European or Zebu of good milk or beef suitability. However, as result of its milk production and maternal skill, their reproductive performance is compromised during the postpartum, which makes the restricted suckling and hormonal therapy important strategies to anticipate the return to ovarian luteal activity.

Material and Methods

Twenty two lactating Indubrasil anestrus cows (74.13 ± 36.15 days postpartum) with 3.38 ± 0.61 body condition score (scale from 1 to 5) were evaluated by rectal palpation and ultrasound exam and submitted to hormonal treatment as follows: Day 0 - measurement of the largest dominant follicle; intravaginal progesterone device insertion + 1mg of Estradiol Benzoate (EB); Day 8 - implant withdrawal + 1mg of Estradiol Cipionate (EC) and beginning of once a day suckling. Estrus detection was done for 30 min twice a day (6:00 e 17:00 h) aided by teaser bulls using chinball markers. Artificial insemination (AI) took place 12 h after estrus detection. Thirty days before the end of 120-days breeding season, cows were placed with intact bull. Pregnancy diagnosis was performed 30 to 45 days after inseminations and at the end of breeding season by rectal palpation and ultrasound exam.

Results and Discussion

Estrus and pregnancy rates were 63.6% (14/22) and 59.0% (13/22), respectively. The interval from EC injection to estrus was 41.8 ± 12.5h, as observed for the other zebu breeds. Non-pregnant cows (41.0%; 9/22) returned to estrus at 27.6 ± 11.6 days and 22.2% (2/9) became pregnant after AI. Induction of ovulation in postpartum anestrous zebu cows after hormonal therapy is possibly associated to the reducing of suckling effect, good body condition score (1) and better estrus detection. The fertility obtained with AI was 72.7% (16/22). The use of intact bull increased 22.7% (5/22) on the final pregnancy rate (95%; 21/22). Estrus and ovulation rate observed on the present study can be due to the follicular diameter (11.3 ± 2.6mm) at the beginning of hormone protocol, which is related to better body condition scores, metabolic status (1) and can have better response to hormonal therapy (2).

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Comparative aspects of the Sigmodontinae yolk sac placenta (*Rodentia, Cricetidae*)

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Introduction
Placentation in rodents is characterized by formation of two different placental membranes. Besides the chorioallantoic placenta that promotes a relationship hemochorial between maternal and fetal blood systems, there is a yolk sac placenta (1). It is a functional placenta in rodents before the formation of the chorioallantoic placenta (2). Moreover, is associated with the transfer of passive immunity from mother to fetus (3) and hematopoietic activity. This research focuses on the characteristics of yolk sac placenta, the functional organ for maternal-fetal exchange in the first stages of placentation, from 5 Sigmodontinae species.

Material and Methods
Placentae from 5 Sigmodontinae species (*Necromys lasiurus*, *Oligoryzomys* sp., *Oryzomys subflavus*, *Oryzomys megacephalus* and *Oryzomys* sp.), were obtained from the Collection of Zoological Museum of Sao Paulo University, Brazil. Four placentae from each species, fixed in 10% formaldehyde, were subjected to histological standard processing for staining with: H&E, Masson's Trichrome and Periodic acid Schiff (PAS).

Results and Discussion
The yolk sac placenta is an inner wall vascularized by vitelline blood vessels (1) and it was observed in the 5 species analyzed. It is a structure formed by endodermic cells that forming several villous. These are present in the placenta fetal side and these are supported by an axis of fetal mesenchyme coated by a simple epithelium with prismatic cells, richly vascularizated by vitellini vessels. Between the villous are presented the blood islands. In placentae of early gestation the villous are more developed than in term placentae. Only the yolk sac placenta of *Oryzomys subflavus* was positive to PAS reaction, and showed extensive haemophagous activity (stained by Masson's Trichrome). The endodermic cells showed a cylindrical shape in the 5 sigmodonts species, but the nucleus position of these cells are located in different regions of the cytoplasm. In *Necromys lasiurus*, *Oryzomys subflavus*, *O.* sp. and *O. megacephalus*, the cell nucleus are located in the cell base, next to mesenchyme axis. And in *Oligorizomys* sp., the endodermic cell nucleus is located in the apical region. These results imply that yolk sac placenta is similar among the sigmodonts species analyzed in this research. Moreover, *Oryzomys subflavus* showed interesting and unique characteristics about the yolk sac placenta.

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Estrous cycle and vaginal cytology in albino Wistar rats: new considerations

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Introduction

The rat has been elected the animal model in diverse studies involving reproduction. However, there are scarce and conflicting data related to its estrous cycle. The duration of estrous cycle is usually 4 to 5 days. It has 5 different phases characterized by cell types occurred in vaginal smears (1,2,3); the phases are: proestrous, estrous, metaestrous I, metaestrous II and diestrous. Nevertheless, this classification and the time-span of each phase are controversial and can induce to misleading interpretations. In addition, there are no reports about cell type quantification in each phase which allow an accurate assessment of the estrous cycle. Thus, the goal of this study was carry out, in female rats, a detailed morphological description of the cell types and to determine the scores of these cell types in each phase of the estrous cycle as well as in the transition periods among the phases.

Materials and Methods

Vaginal smears were obtained from 10 Wistar rat females, twice daily and during 20 consecutive days. Due to the short time-span of the estrous cycle phases and with the aim to better evaluate and classify theses phases, daily collections were performed with an interval of 8 hours. Vaginal smears were stained using Shorr method. Morphometric analyses were carried out from these smears using a square lattice eyepiece as test-system, which was connected to a light microscope. Cells were classified as small and large basophilic cells, nucleated and enucleated acidophilic cells, transition cells (with heterogeneously stained cytoplasm) and leukocytes. Ten fields in each smear were randomly analyzed at X400 magnification and the cellular frequencies were determined and expressed as percentage. A statistical analysis was performed using one way analysis of variance (ANOVA). The multiple comparison test (Student-Newman-Keuls method) was also used when the results showed statistical significance.

Results and Discussion

Size and cellular morphological characteristics, cytoplasm-nucleus ratio and staining properties were in according to previously described (1). However, blue and dark-orange stained cells were considered as transition cells. Figure 1 shows the scores of the cell types in each stage of estrous cycle. Enucleated acidophilic cells were observed in all phases. Occurrence of transition cells was high in proestrous, but these cells were not found in the estrous. Nucleated acidophilic cell frequency was higher in the transition period (between the metaestrous II and diestrous) than in other phases. These data can be useful to distinguish the two latter phases. Leukocytes were first observed in the metaestrous and showed very high frequency in the diestrous. Finally, our study emphasizes the quantitative analysis of the cellular populations in the vaginal smear and provides additional information for a reliable classification.

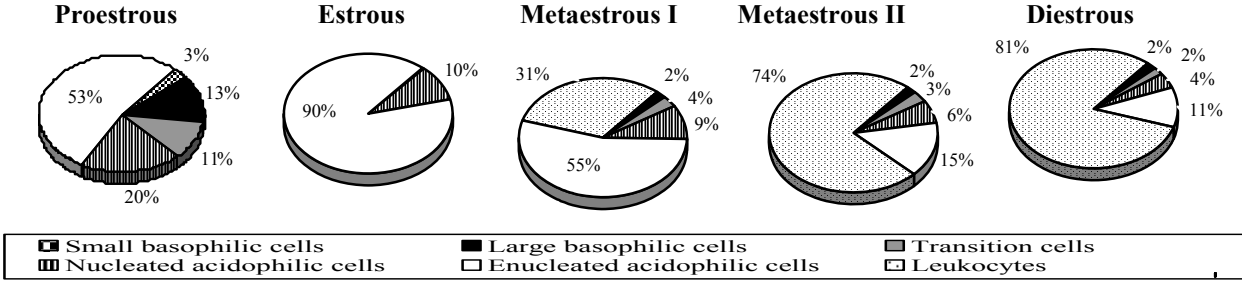

Figure 1. Frequencies of the cell types in each phase of the estrous cycle, expressed as percentage.

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Morphological changes in the umbilical cord of mice fetuses exposed to high levels of urban particulate matter during pregnancy.

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Introduction

Adverse pregnancy outcomes (LBW, IUGR) are associated with exposure to air pollution. Recently we have demonstrated (in mice) that low birth weight and changes in placental functional morphology are associated to chronic exposure to urban particulate matter during gestation (1). Compromised fetal blood flow through the umbilical cord vessels can have deleterious effects on fetal health. Several studies have shown that morphologic changes of the umbilical cord are associated with adverse perinatal outcomes such as IUGR (2). We hypothesized that the effects of urban air pollution on pregnancy outcomes could also be related to changes in morphology of the umbilical cord (UC).

Materials and Methods

Mice were maintained for two generations (G2) in exposure chambers situated close to a busy street of traffic in São Paulo. Two groups of G2 (n = 6) females were raised and completed pregnancies in normobaric chambers with exclusively filtered (F) or non-filtered (NF) air. In NF chambers, three filters eliminated different sizes of particulate matter but not gaseous pollutants. The 24-hr concentration of particulate matter with a diameter of less than 2.5μm ($PM_{2.5}$) was determined gravimetrically. At 18-days gestation, female mice were euthanized and 1 UC from each female removed, fixed in formalin, embedded in paraffin and stained for microscopical examination. Fields of view on cross section slices were analyzed stereologically using Cavalieri principle. Volumes and mean areas of UC compartments were estimated. Group comparisons were drawn using the Mann-Whitney U-test. Null hypotheses were rejected at $P < 0.05$.

Results and Discussion

Concentrations of $PM_{2.5}$ in F chambers were significantly lower (76%, $P < 0.001$) than those in NF chambers. Results of umbilical cord morphology are summarized in the table below:

Compartment	Total volume (μm³)		Lumen volume (μm³)		Wall volume (μm³)		Sectional area (μm²)		Lumen area (μm²)		Wall thickness (μm)	
	F	NF	F	NF	F	NF	F	NF	F	NF	F	NF
umbilical cord [a]	1.47 (0.1)	1.11 (0.1)*	-	-	-	-	0.27 (003)	0.2 (0.01)*	-	-	-	-
allantoic artery	0.12 (0.02)	0.15 (0.04)	0.035(0.01)	0.043 (0.01)	0.09 (0.01)	0.1 (0.02)	11014.5 (1768)	9383.8 (2477)	3042.4 (923)	2613.4 (747)	28.2 (2.9)	25.3(3.8)
allantoic vein	0.11 (0.01)	0.14(0.02)*	0.067 (0.01)	0.048 (0.01)*	0.047 (0.007)	0.09 (0.02)*	10191.7 (2706)	8154.7 (2830)	6058.3 (2233)	2774.5 (1342)*	13.2 (1.7)	21.2(5.2)*
vitelline artery	0.06 (0.03)	0.04 (0.02)	0.033 (0.02)	0.024 (0.01)	0.028 (0.01)	0.023 (0.01)	6267.4 (1170)	3596.7 (956)*	3223.2 (1559)	1686.3 (861)	13 (2.3)	10.9(4.6)
vitelline vein	0.15 (0.04)	0.13 (0.02)	0.093 (0.01)	0.057 (0.03)*	0.057 (0.03)	0.07 (0.01)	11193.7 (2072)	8576.9 (4061)	7363.4 (2243)	3905.7 (2431)	11.5 (2.8)	17.2(3.5)*
mesenchyme [a]	1.02 (0.1)	0.66 (0.04)*	-	-	-	-	-	-	-	-	-	-
vessels [a]	0.4 (0.08)	0.47 (0.08)	-	-	-	-	-	-	-	-	-	-

$p < 0.05$; [a] consider mm³ (volume) and mm² (area) as metric unit

Findings indicate that UC morphology is affected by particulate urban air pollution. Data suggest changes affecting mesenchyme and also veins rather than arteries. Reductions in UC volume and diameter, increases in vessels wall thickness followed by reduction in luminal areas may be linked to altered uteroplacental resistance and haemorheology.

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Uterine and vaginal fibrosarcoma in female dogs – a report on five cases

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Introduction

Fibrosarcoma is a rare tumor in mesenchymal cells. It originates from malignant fibroblasts and is characterized by the interlacement of collagen fibers. It may occur in various tissue types, such as those in the bladder, liver, urethra, lungs, trachea, bones as well as in cutaneous tissue and in the oral and nasal cavities. It is rarely found in the genital system (2). This sarcoma also develops from injection sites in dogs and particularly in cats (3). It may occur in any breed; however, it is more commonly found in female dogs and cats at approximately 9 years of age. In most cases, fibrosarcoma presents as a lobulated sessile structure with whitish gray coloration, it is non-hemorrhagic and painless, showing irregular depression indicative of necrosis. It may have a circumscribed capsulated aspect or not; or an infiltrative character of rapid development which causes the destruction of the affected tissue and is similar to an ulcerated pyogenic granuloma, granuloma of giant peripheral cells or peripheral bone fibroma (2). It grows spontaneously, and its cause has not been identified, but it may be associated with genetic mutations, such as the loss of alleles and points of chromosomal mutation and translocation. It presents a high rate of pulmonary metastasis with a survival period of less than five years (2). Surgical resection is the elected treatment for these sarcomas, in addition to adjuvant chemotherapy and radiotherapy (2).

Cases Report

Five female dogs of the breeds Doberman, Cocker Spaniel, Poodle and NSB were attended to at the Veterinary Hospital of FCAV/UNESP. They were 6 to 12 years old and presented vaginal fibrosarcoma ranging from 0.5 to 11cm. Of these five dogs, three developed metastasis in the body of uterus (1 to 6cm), two showed mixed malignant mammary tumors and one had metastasis in the lung and adrenal gland. After performance of laboratory tests, the animals were submitted to ovariohysterectomy, episiotomy and vulvovaginoplasty, in addition to unilateral mastectomy, adrenalectomy in the NSB and nodulectomy in the Poodle. Microscopic examination showed interstitial hemorrhage, accentuated cellularity and pleomorphism, proliferation of neoplastic cells from immature fibroblasts and interlaced collagen fibers, elongated and fusiform cells, elongated and hyperchromatic nuclei, eosinophilic cytoplasm, infiltrative character in the Cocker Spaniel and NSB and capsulated in the Poodle and Doberman. After surgery, the animals were submitted to antiblastic therapy following the VAC II protocol (1mg/kg IM of Diphenhydramine prior to the administration of 30mg/m^2 IV of Doxorubicin and 200mg/m^2 of Cyclophosphamida IV or VO on the 1st day and 0.75mg/m^2 IV of Vincristine on the 8th and 15th days). The chemotherapeutic protocol can be repeated from 4 to 6 times, according to the oncologist's evaluation. The animals are presently in excellent health conditions and have been periodically checked in order to discard tumor relapse.

Results and Discussion

Johnston (1998) states that vaginal tumors are induced by hormones, and uterine tumors are related to estral irregularity, but their occurrence in ovariohysterectomized dogs and in those with a regular estral cycle is an argument against such hypothesis. In this report, all the animals were entire females presenting estral-cycle regularity. Primary tumors in the mammary gland may develop metastases in the uterus and the vagina (3), and vaginal and uterine tumors may develop metastases in regional lymph nodes, ovaries, adrenal glands, thyroidal glands and lungs (1). Three dogs in this report presented uterine and/or vaginal neoplasia associated with neoplasia in the mammary gland, and one presented metastasis in the adrenal gland. Surgical resection is the elected treatment for these metastatic sarcomas in soft tissues, with support from adjuvant chemotherapy and radiotherapy, and the rapid diagnosis and treatment of such neoplasias are fundamental to improve prognosis and increase the patient's survival.

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Morphologic and functional study of the type II pneumocyte related with the gestational age in bovines (*Bos taurus* and *Bos indicus*)

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Introduction
The objective of this study is to characterize the presence of type II pneumocyte and the beginning of the surfactant protein production in bovines, correlated with the gestational age.

Materials and Methods
Twenty eight fetuses in gestational age between 4-8 months (collected from slaughterhouses in São Paulo), and two newborn animals (euthanized after birth by overdose of anesthetic, in accordance to the USP Bioethical Committee guidance) were weighed and measured, using the Crow – Rump (CR) method. For the morphologic description, pulmonary fragments of 2cm² each were collected. The fragments were fixed in Bouin solution for light microscopy and in glutaraldehyde 2.5% for transmission electron microscopy. For the biochemistry study, incisions and the introduction of a catheter were done in the trachea area and pulmonary wash was carried out with physiological saline solution (0.9%).

Results and Discussion
Lung of fetuses with gestational age of 4 months were found in the canalicular development phase, without the presence of globule cells, or appearance of electroforetic bands compatible with the presence of surfactant proteins. In fetuses 5 months old the lungs were in terminal sac phase, with the presence of primitive alveolus, formed by cubical epithelium, with areas formed by pavimented cells and globule cells. In the analysis of electroforese surfactants, proteins had not been identified. Development of terminal sac phase in the lungs of fetuses of 6 months old were observed, with the presence of type I and type II pneumocytes. In this phase the SDS-PAGE analysis showed the presence of bands about 28 kDa, demonstrating SP–A production, which was the surfactant protein found in greater amount. From 7 months old and older the phase of terminal sac was more evident and complex, with the sprouting of intense vascularization. The type I pneumocyte had a more pavimented aspect and type II was more globular. In the SDS–PAGE analysis of the bronchial – alveolar wash, surfactant protein bands had been observed with similar profile from that of the newborn animals. In newborn animals, lung in the alveolar phase was observed, with the type I and II pneumocyte already defined. The profile of the bronchial–alveolar wash from the newborn was the same as that of an adult animal, presenting the same bands in the triplet. The surfactant proteins production was found from the 6th gestational month, when only the SP–A protein have been found; however it was in the 7th month that all surfactant proteins (SP–A, SP–B, SP–C and SP–D) were produced with a similar electroforetic profile to the adult, allowing a complete respiratory function in the event of a premature birth.

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Expression of the vascular endothelial growth factor-A (VEGF-A) and basic fibroblast growth factor (bFGF) and their receptors in placentomes from cloned bovines fetuses

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Introduction

The unsatisfactory efficiency of the somatic cell cloning technique has been associated with placental abnormalities especially alterations of the vascular architecture (1). Although 5% of cloned cattle gestations come to term, uterine/placental environment plays an important role determining offspring survival rate (1, 2, 3). An aberrant gene expression in placentomes has been suggested as one of the major factors contributing to maternal/fetal altered exchanges (4, 5). Since placental growth is largely dependent on the preceding or concomitant growth of blood vessels (6) and VEGF-A and bFGF are the most important growth factors controlling neovascularization and vascular permeability in the placenta (7, 8) we hypothesize that the VEGF-A and bFGF systems are disrupted in placentomes from cloned animals, thus contributing to placental abnormalities. Therefore, the objective of this study was to determine mRNA expression and cellular localization of VEGF-A, bFGF and their receptors in placentomes from cloned bovine fetuses.

Material and Methods

Placentomes from cloned (n = 8; 4 males and 4 females) and non-cloned (n=4) bovines fetuses were collected after cesarean sections at term (270 days). Bovine clones were produced by somatic cell nuclear transfer as described before (9). Placentomal tissues were either fixed in 4 % phosphate buffered formalin solution for immunohistochemical analysis or immediately frozen in liquid nitrogen and kept were -80 ºC until RNA extraction. Relative gene expression of VEGF-A, VEGFR1, VEGFR2, bFGF, FGFR1, FGFR2, FGFR3 and FGFR4 was assessed by real time RT-PCR, using GAPDH as endogen control gene. Cellular localization of proteins codified by these genes was determined following immunohistochemistry.

Results and Discussion

Real-time PCR revealed that mean levels of VEGFR-2 mRNA were significantly higher in male placentomes compared with non-cloned animals while bFGF and all studied receptors were decreased in placentomes from female cloned fetuses compared with males and non-cloned animals. Although bFGF system showed a decreased expression in female clones, these animals had a relative higher VEGF expression when compared with males and non-cloned fetuses. VEGF-A and its receptors proteins were localized in placentomal endothelial cells as well as in maternal and fetal epithelial and stromal cells. In addition, immunolocalization of VEGF-A system in the placentome demonstrated a variable pattern of protein cellular distribution comparing either cloned and non-cloned animals or clones among them. Basic FGF and receptors were less expressed in the cloned bovine fetuses and their cellular distribution showed also a distinct pattern among cloned and non-cloned bovine placentomes. These differences in placental gene expression may be related with an abnormal epigenetic status in the organ. Many reports have described aberrant DNA methylation and altered patterns of gene expression for several imprinted and non-imprinted genes, including VEGFR-2, in the placenta (4, 5, 10). Furthermore, the decrease of bFGF and FGFRs expression may be a consequence of bFGF system disruption in the placenta from female fetuses, which results in an increase of VEGF expression in placentomes. The modulatory role of bFGF on VEGF and VEGFRs expression has been already described in the corpus luteum (11, 12) and determines the ideal imbalance of angiogenic factors and vascularization progress. Alterations in VEGF-A and bFGF systems expression suggest that angiogenic factors may be involved in abnormal placental development in cloned gestations contributing to pregnancy loss, fetal and placental development aberrations and/or poor survival of neonates.

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Gestational length and offspring sex ratio of mares bred by Pega jacks (*E. asinus*)

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Introduction
Mares are commonly bred by jacks in several countries in the world including Brazil, Cuba, United States, Mexico and Argentina (1). The Pega jack has been largely used for mule production in several Brazilian states (1). Mules are appreciated in rural areas because of their strength and endurance in herding beef cattle in large farms and in general agricultural activities such as traction and transport. The length of gestation in mares is highly variable (2) and prediction of parturition is essential in the general management of the stud farm and in organizing assistance during the foaling. It is widely believed by owners of mule stud farms that the majority of mule foals are male. Female mules are prized over six times the value of male mules so offspring sex ratio is of great importance to breeders.The objective of this study was to describe the gestation length and offspring sex ratio of mares bred by Pega jacks during ten reproductive seasons.

Material and Methods
Retrospective data evaluated in this study involved 54 pregnancies in 43 crossbreed mares (Breton, Campolina, Margalarga Marchador) from 1983 to 1994. The animals belonged to the Equine Breeding Centre – Department of Animal Science, Federal University of Viçosa, (20º45'20''; 42º52'40''). The mares were kept in native and semi cultivated pastures (*Melinis multiflora*) and had free access to mineral salt. During the drought season the mares were supplemented with fresh cut grass (*Pennisetum purpureum*). The mares were bred to eight Pega jacks by natural breeding or artificial insemination. Gestation length was calculated from the day of the last breeding. All mares had normal, non-induced parturition and delivered normal mule foals. The effect of the male on gestation length and offspring sex ratio was analyzed using the Kruskal-Wallis test and Chi square test with the Statics Analysis and Genetics Program (SAEG, 2007- UFV).

Results and Discussion
Gestation length was 334.35 ± 10.3 days (mean ± SD) and no significant statistical differences were observed among the jacks on gestational length. Offspring sex ratio was 48.18% (26/54) male mules and 51.85% (28/54) female mules. The distribution of the sex ratio during the seasons was variable, but no significant differences were observed between the sexes. The gestation length observed in the present study was similar to that for mares bred by Thoroughbred (2) and Crioulo (3) stallions. In conclusion, the length of gestation of mares bred by Pega jacks in Minas Gerais State-Brazil was not affected by individual jacks and was similar to the average observed in mares bred by stallions. In addition, similar proportions of male and female mules were carried to term. Additional studies should investigate the gestation length of mares bred by jacks in different conditions and include a greater number of records.

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Embryos ultrastructure from Alpine goats (*Capra hircus*) supplied with urea in the diet

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Introduction
Ruminants can use urea for synthesis of microbial protein, allowing a partial replacement of protein concentrates from the diet by urea. However, the urea supplement on ruminant nutrition has been related to an increased concentration of N-urea in the blood, reduction in uterine pH (1) and in the embryos viability and development (2). The objective of this study was to evaluate the effect of the urea supplementation in the diet on the embryonic ultrastructure.

Material and Methods
Twenty-two goats were submitted to four treatments: 0.0% (T1), 0.73% (T2), 1.46% (T3) and 2.24% (T4) of urea, respectively, in the dry matter of the diet for 81 days before the beginning of the experiment. For ultrastructural analysis, two grade I expanded blastocysts from T1, four grade I expanded blastocysts of each one from T2 and T4 and three expanded blastocysts of grades I and II from T3 were prepared for transmission electron microscopy.

Results and Discussion
Trophoblast cells (TC) from T1 embryos were flattened, less electron-dense and polarized with abundant microvilli in their apical surface. Lipid droplets associated to the mature or immature mitochondria were observed. TCs were joined to each other by junctional complex. The perivitelline space (PVS) was narrow and occupied by the TCs microvilli. Sparse extruded material was observed in the PVS. TCs from T2 embryos were similar to those observed in the T1 embryos, except by the presence of electron-dense inclusions in contact with the zona pellucida (ZP) and autophagosomes in the cytoplasm. Inner cell mass (ICM) showed large spaces containing degenerating cells. One embryo from T3 showed similar cellular morphology to those observed in the T1 and T2 embryos; however, the cytoplasm of the TCs showed different features regarding to disposition of the organelles, cytoskeleton structure, increased number of autophagosomes and myelinic figures. The other two embryos from T3 showed intense cellular morphological modifications. TCs showed a globular shape with few microvilli or showed as cytoplasm portions very flattened depleted of microvilli. TCs cytoplasm showed many vacuoles, autophagosomes, lipid droplets, few organelles and cytoskeleton elements. The PVS was enlarged, with cells debris and extruded blastomeres. Cellular debris were also observed in the blastocoele. Degenerating cells were more common in the trophoblast epithelium of T3 embryos. All embryos from T4 were lost during the electron microscopy methodology. The results suggest that high urea concentration in the diet may cause embryo cellular modifications related to cell reorganization and/or degeneration.

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Seasonal influence in biological indexes of piapara (*leporinus obtusidens*) captured at downstream of a hydroelectric dam in the Grande river, Minas Gerais

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Introduction
Piapara (*Leporinus obtusidens*) as the majority of the species of tropical fish, migrate upward the river on the onset of the reproduction period (November to January). The displacement of hundreds of kilometers, during the migration, affects all the physiology of these fish, unchaining essential processes for the preparation of reproduction. The construction of hydroelectric dams has influenced the reproduction of the migratory species since they are restrained to carry through the migratory behavior that hinders them to reach the physiological preparation for the reproduction. The objective of this study is to evaluate the reproductive characteristics, through biological indexes, of piapara captured downstream of the hydroelectric dam in the Grande river, during a reproductive cycle.

Materials and Methods
The piapara were captured downstream of a hydroelectric dam in the Grande river in the period of September, 2006 to August, 2007. The collections were monthly made with a total of 127 animals captured. The fish were measured and weighed. Their gonads and liver were removed and weighed for calculation of the biological indexes: gonadosomatic (GSI) and hepatosomatic (HSI). The fish samples, collection time and biological indexes in both males and females were evaluated through a statistical program R version 2.7.1.

Results and Discussion
Table 1 shows the biological indexes under study, the gonadosomatic index (GSI) and hepatosomatic (HSI). In the females during the spring the GSI was significantly higher than in summer and autumn, and similar to the winter, while no differences were observed among seasons for the males. Furthermore, it was also noted that in spring these indexes were significantly higher in relation to the other seasons. This increase in GSI is compatible with the "piracema" period, when the fishes are reproducing because of the climatic variations (1) and their gonads were replete of gametes. In regard to the HSI, there was a significant difference between spring and summer compared with autumn and winter, with the first showing a higher index. These results indicate that the process of gonadal maturation was started but not completed as evidenced by high values of HSI found in spring and summer. Thus, especially the females were affected in some way by the changes of the river.

Table 1. Means (±SD) of gonadosomatic (GSI) and hepatosomatic (HSI) indexes of *Leporinus obtusidens*, during a reproductive cycle.

Seasons	GSI		HSI
	Females	Males	(females and males)
Spring	1.828 ±1 .43A	0.609 ± 0.07A	0.70 7± 0.14A
Summer	0.513 ± 0.09C	0.102 ± 0,04B	0.714 ± 0.16A
Autumn	0.596 ± 0.09BC	0.065 ± 0.02B	0.532 ± 0.08B
Winter	1.075 ± 0.82AB	0.173 ± 0.11B	0.551 ± 0.05B

*Means followed by different letters within columns differ significantly (p>0.05) by Tukey test.

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Canine male pseudohermaphroditism: case report

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Introduction
The true hermaphrodites are individuals with testicular tissue and ovarian combined in one unique gonads (ovotestis) or present in separated gonads (1). Pseudohermaphrodites subjects feature a singular type of gonadal tissue that agrees to the chromosomal sex, but disagrees to the gonadal sex (4), so they are classified in male or female (1). The pseudohermaphroditis female possesses ovaries and present some degree of masculinization; whereas the males possesses testicles and show female characteristics. Two categories of male pseudohermaphroditism are recognized: the persistent Müllerian duct syndrome (PMDS) and failure in the androgen-dependent masculinization process. In the PMDS, dogs are capable to produce the duct Müller inhibition (MIS) in the embrionary period, on the other hand a defect in the MIS receptors become the tissue without respond to it (3). The failure in the masculinization process can be partial or complete depending if the androgen receptor are partially or completely functional (4), independent on the androgens concentration (2). The affected animals possesses bilateral testicles, normally cryptorchid, external female genitalia, no evidence of internal derivate from Müller ducts (4) or from structures derivate from Wolff ducts (2). The pseudohermaphrodites diagnoses must be based on the chromosomes inspection, gonads and in the phenotype appearance from the reproductive organs. Therefore, the main objective of this research is to present the male pseudohermaphroditism in dog, the complementary exams performed to diagnoses and the therapeutic procedure adopted.

Case report
The present report describes a one year-old German Sheppard that was considered as a female, but presented a male behavior, absence of a normal estrus cycle, and an abnormal structure in the vagina. In general physical examination a normal vulva and vaginal tract were noticed and also a hemipenis containing penile bone, bulb and glans was presented at the clitorian cavity. The urethra was located at 1 cm caudal to the vaginovestibular junction, like normal females. Routine hematology, biochemistry and cytogenetic analysis were ruled out, as well as hormone concentration. Radiography and ultrasonography showed both uterus and testicles in the abdomen, and the presence of a penile bone. Hysterectomy and orchidectomy were performed and specimens were evaluated by histopathology. Additionally, episiotomy and vulvovaginoplasty were carried out. The uterus, sustained by the mesometrium, was composed from uterine cervix, uterus body and uterines horns. The two testicles were connected to the peritoneum by the cremaster muscle and pampiniform plexus, situated caudal to the kidneys, in the anatomic position of ovaries of normal females; the ligament from the tale to the epididymis and the testicle ligament connected the epididyms from both testicles of uterines horns. Episiotomy was performed to remove penis-like structure and vulvoplasty to the vagina reconstruction. Cytogenetic analysis revealed a 78, XY; histopathology confirmed the presence of the two testicles and uterus, hormone concentrations were: 0.22ng/ml of progesterone, 34.4 pg/ml of 17-β estradiol, and 242ng/ml of testosterone.

Discussion
It was observed that the treated animal present agreement between the chromosomal sex and the gonadal sex. Despite the fact that the external genitalia was phenotypically female, the gonads were male, what allowed to classified it as male pseudohermaphrodites (4). Considering that the animal presented Muller ducts components (uterus and vagina) and Wolff ducts components (epididymis, deferent duct, seminal vesicle and ejaculatory duct), the diagnosis of male pseudohermaphroditism due to persistent of the Müllerian duct syndrome was concluded (3).

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Changes in biological indexes of dourado (*Salminus Brasiliensis*) collected at downstream of a hydroeletric dam in the Grande River, Minas Gerais

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Introduction

Many factors cause impact on populations of native migratory Brazilian fish. Hydroelectric dams cause major changes in habitat of the river, together with the interruption of the migratory processes both for food and for reproduction. The dourado (*Salminus brasiliensis*) is considered one of the largest and most noble of Brazilian fish fresh water (1). It is "piracema" species, not reproducing in lake environment. For this reason the hydroelectric dams carry great influence on the biology of this species. The objective of this study is assessing changes in the biological indexes of *Salminus brasiliensis* captured at downstream of Funil Dam in the Grande river, during a reproductive cycle.

Materials and Methods

The fish were monthly captured downstream of Funil Dam in the Grande river in the period of September 2006 to August 2007. A total of 53 animals had been captured. The fish were measured and weighed. Their gonads and liver were removed and weighed for calculation of the biological indexes: gonadosomatic (GSI) and hepatosomatic (HSI). The fish samples, collection time and biological indexes in both males and females were evaluated through the statistical program R version 2.7.1.

Results and Discussion

For females, the GSI presented the lowest value in summer, opposite behavior was observed for the HSI, which presented the highest value in that same season. For males, the GSI was higher in spring. These results indicate that during the reproductive cycle studied, the females have started the gonadal maturation, but have been impaired in some way in the final process of maturation, which includes the vitelogenic process of oocytes. This was confirmed by increased of the HIS in summer, indicating a metabolic process of reabsorption of female's gametes.

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Rate between germ cells and support cells during postnatal development of the testis in the wild boar (*Sus scrofa scrofa*)

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Introduction

Despite the intense exploitation of the commercial potential of the wild boar observed during the last years, few studies have investigated its reproductive biology. The spermatogenic process has been described in adult wild boars (1), but there are no reports on cell proliferation rates during the post-natal development of these animals. The present study aimed to analyze the rate of germ cells and support cells in wild boars, during the period from birth to 12 months of age.

Materials and Methods

Thirty-nine captive male wild boars were grouped according to age, from birth to 12 months of age, and analyzed monthly, resulting in 13 experimental groups. Animals were submitted to unilateral orchidectomy. Fragments of the testicular parenchyma were prepared to histological analysis (1). Cell populations present in the seminiferous epithelium were determined by analysis of at least 10 transversal sections of seminiferous chords or seminiferous tubules (in stage 1 CSE). The rate of germ cells and support cells was determined by the analysis of the ratios between: gonocytes (G):support cells (undifferentiated support cells and Sertoli cells - SC); spermatogonias (SPTG):(SC); primary spermatocytes in pre-leptothene/leptothene (PL/L):(SC); primary spermatocytes in pachytene (PC):(SC); round spermatids (RS):(SC); and total germ cells (GC):(SC).

Results and Discussion

The rate between SC:GC was variable in the pre-pubertal stage, probably due to the varying numbers of undifferentiated support cells observed in this period. In pubertal animals, at around nine months of age, Sertoli cells showed an increase capacity of support, due to the stabilization of proliferation and simultaneously to the increased number of PL/L (table 1). The index derived from the rate of Sertoli cells and RS in 12-month-old wild boars was lower than that observed in Piau boars (2). Lower indices in wild animals may be related to the selective process used with domestic boar, which probably has resulted in greater efficiency of Sertoli cells. Similar results were observed for the index of Sertoli cells relative to the total population of germ cells, which was lower than that of Piau pigs (2). We conclude that rate of support cells and germ cells increased from puberty to 12 months of age.

Table 1. Rate between number of support cells and germ cells in wild boars from birth to 12 months.

Age (months)	SC:G*	SC:SPTG*	SC:PL/L*	SC:PC*	SC:RS*	SC:total GC*
0	1 : 0.14	-	-	-	-	1 : 0.14
1	1 : 0.08	-	-	-	-	1 : 0.08
2	1 : 0.06	-	-	-	-	1 : 0.06
3	1 : 0.06	1 : 0.08	-	-	-	1 : 0.14
4	-	1 : 0.12	-	-	-	1 : 0.12
5	-	1 : 0.14	-	-	-	1 : 0.14
6	-	1 : 0.11	-	-	-	1 : 0.11
7	-	1 : 0.15	-	-	-	1 : 0.15
8	-	1 : 0.22	1 : 0.03	-	-	1 : 0.25
9	-	1 : 0.23	1 : 0.93	1 : 1.12	1 : 3.35	1 : 5.63
10	-	1 : 0.21	1 : 1.16	1 : 1.34	1 : 4.06	1 : 6.77
11	-	1 : 0.19	1 : 1.28	1 : 1.18	1 : 3.61	1 : 6.26
12	-	1 : 0.18	1 : 1.80	1 : 2.00	1 : 5.58	1 : 9.56

*Numbers calculated according Abercrombie method (3): gonocytes (G), support cells (SC); spermatogonias (SPTG), spermatocytes I in pre-leptothene/leptothene (PL/L), spermatocytes I in pachytene (PC), round spermatids (RS), germ cells:(GC).

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Previous purification of seminal plasma proteins from goats through Sephadex G-25 and G25-80

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Introduction

The study of seminal proteins is considered one important tool to be used in our days aiming to select genes connected to fertility or others characteristics. In some species these studies are advanced, but the same does not happens with goats. Aiming to improve the investigation on proteins bands with low molecular weight from Anglo-Nubian goats seminal plasma, it were used the ion change chromatography, Sephadex columns G-25 and G25-80 before to use the one dimension electrophoresis SDS-PAGE.

Materials and Methods

Semen collections were made weekly during the rainy (April – May) and dry (October – November) periods from five Anglo-Nubian goat thorough artificial vagina and it was used a pool from these collections. The Sephadex G-25 and G25-80 columns were eluted in 1: 25 phosphate buffer. During the run column it were collected six samples of 1 mL and after that realized the total proteins determination (Bradford, 1976) whose medium valor was 0.32 ug/ul for Sephadex G25, but it was not possible to calculate a medium value to G25-80 due to presence of no homogeneous peaks. The protein picks observed after Sephadex columns were submitted to one dimension electrophoresis 7.5% SDS-PAGE.

Results and Discussion

The protein peaks observed after Sephadex G-25 and G25-80 are on Figure 1. The gel analyze are showed on Table 1, where it is possible to observe bands from 70 kDa to 54 kDa. Studies with goat seminal plasma proteins without use of Sephadex column presented proteins bands variation from 13 kDa to 150 kDa (Teixeira, 2008), range that becomes difficult the protein study. Taken into consideration that this method is easy to perform and of low cost we recommend it to seminal plasma proteins bands isolation. Also, the gel G25 showed to be better to identify peaks concentration.

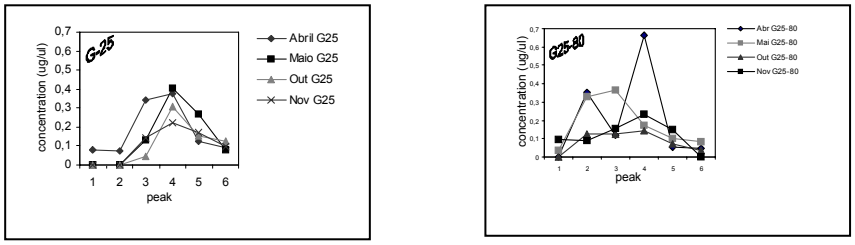

Figure 1. Peaks observed after Sephadex G25 and G25-80 columns in goats seminal plasma.

Table 1. Proteins bands (kDa) observed at 1D gel electrophoresis 7.5% SDS-PAGE, in goat seminal plasma samples after Sephadex run column.

Bands	Sephadex G-25				Sephadex G25-80			
	Apr	May	Oct	Nov	Apr	May	Oct	Nov
1	70	70	70	70	70	70	70	70
2	59		65		60		65	
3			59		54		59	

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Sperm damage evaluation caused by separation of bovine X-bearing sperm by centrifugation in discontinuous Percoll™ density gradient using fluorescence probes association

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Introduction

The main importance of sex selection is established in farm animals where the productivity is enhanced by progeny of one sex. Satisfactory results are being achieved in our laboratory utilizing Percoll™ gradients for X-bearing sperm separation in bovines, employing a simple and cheap methodology (1). However, the benefits are observed only if the sex selection technique does not reduce the reproductive potential of semen. Hence, we evaluated the effects that the separation of X-bearing bovine sperm by centrifugation in discontinuous Percoll™ density gradient has on sperm viability parameters utilizing the fluorescent probes association. Thus, we aimed to collect truthful information about the sperm differences before and after this sexing process.

Materials and Methods

Frozen-thawed semen samples from six different bulls (four different batches from each bull) were evaluated before (control group) and after (sexed group) centrifugation in discontinuous Percoll™ gradient. This experiment was designed to assess sperm motility by computer-assisted semen analysis (CASA), morphological characteristics by differential interference microscopy and simultaneous evaluation of the plasma, acrosomal and mitochondrial sperm membranes, using the fluorescent probes: propidium iodide (PI), fluorescein isothiocyanate–Pisum sativum agglutinin (FITC-PSA), and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), respectively (2).

Results and Discussion

The fertility sperm parameters described in Table 1 indicates that the sexed group demonstrates an improvement in the semen quality after sexing procedure, except for the percentage of cells with Intact Acrossome Membrane which was reduced probably due to the capacitation and acrosome reaction phenomenon induced by the selection method.

Table 1. Sperm characteristics from frozen-thawed semen of six different bulls (mean ±sem) before (control) and after (sexed) the X-bearing sperm separation by centrifugation in Percoll™ gradient.

Semen Analysis	Parameters	Control (%)	Sexed (%)
CASA	Total motility	69.9 ± 1.7 [a]	85.0 ± 1.8 [b]
	Progressive Motility	59.2 ±1 .6 [a]	76.0 ± 1.7 [b]
	Linearity	55.5 ± 1.2 [a]	61.4 ± 1.4 [b]
Morphology	Major defects	13.0 ± 1.2 [a]	5.3 ± 1.1 [b]
Fluorescent Probes	Intact Plasma Membrane	59.1 ± 2.2 [a]	72.3 ± 2.1 [b]
	Intact Acrosome Membrane	85.0 ± 1.7 [a]	45.1 ± 2.2 [b]
	High Membrane Potential	77.1 ± 2.0 [a]	87.0 ± 1.7 [b]
	Intact plasma membrane, intact acrosome and mitochondrial function	59.0 ± 2.2 [a]	38.2 ± 2.3 [b]

Different superscript letters in the same line indicate statistical differences (P<0.05).

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Influence of bovine subspecies in the recovered rate after the separation of X-bearing sperm by centrifugation in discontinuous Percoll™ density gradient

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Introduction

The sex selection procedure must not decrease the semen reproductive efficiency to facilitate the commercial applicability of sexed sperm. Another disadvantage of the sex selection techniques is the low number of recovered cells after the X-bearing spermatozoa separation process. Like this, it is important to find new strategies to improve the biotechnology success. The main objective of this work was to evaluate differences between subspecies in the recovered rate after the separation of X-bearing bovine sperm by centrifugation in discontinuous Percoll™ density gradient, that is capable to separate X and Y-bearing sperm with accuracy about of 70% (1, 2).

Materials and Methods

Bovine frozen semen samples of six different breed were evaluated (three Bos taurus and three Bos indicus) and in order to obtain more precise individual data, we evaluated four different batches from each bull. Nine straws from each batch were thawed and homogenized (2.25 ml). To separate X-bearing sperm, 1.5 ml of frozen-thawed semen was split into 0.5 ml aliquots, placed on top of Percoll™ discontinuous density gradient tubes and centrifuged at 500 x g for 20 min at 22° C. The supernatant was aspirated and the sperm pellets were homogenized and collected from the tubes with the last 100 µl Percoll™ medium. Another sample was submitted to sperm concentration (Neubauer chamber) and morphological analysis (differential interference-contrast microscopy), as well as computer-assisted semen analysis (CASA). The three centrifuged sperm pellets were pooled and prepared for the same evaluations. In this way, the semen samples were assessed immediately post-thawing (control) and after centrifugation on Percoll™ density gradients (centrifuged). The sperm recovery rate (RR) was calculated using the equation: RR = [(final volume X final concentration)/(initial volume X initial concentration)] X 100.

Results and Discussion

The influence of bovine subspecies in the separation of X-bearing sperm by centrifugation in Percoll™ gradient is described in Table 1. The Major Defects (MD), Progressive Motility (PM) and Total Motility (TM) results indicates that semen quality was improved after the sexing procedure (centrifuged samples) without subspecies influence. In other hand, it was observed a significant difference between subspecies in the RR.

Table 1. Sperm characteristics and recovered rate (%) from thawed semen of six different bulls (mean± sem) before (control) and after (centrifuged) the X-bearing sperm separation by centrifugation in Percoll™ gradient.

Bovine subspecies	Control *			Centrifuged*			Recovered rate**
	MD	PM	TM	MD	PM	TM	
Taurine	12.0 ± 1.6[a]	59.0 ± 1.9 [a]	69.4 ± 2.6 [a]	4.0 ± 0.9 [b]	78.7 ± 2.2 [b]	86.6 ± 2.5 [b]	6.53 ± 0.8 [c]
Zebuine	14.0 ± 1.9[a]	59.5 ± 2.7 [a]	70.3 ± 2.4 [a]	6.6 ± 2.4 [b]	73.1 ± 2.3 [b]	83.3 ± 2.5 [b]	4.41 ± 0.4 [d]

*Different letters in the row = $p < 0.05$; **Different letters in the column = $p < 0.05$

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Testosterone levels in seminal plasma related with evaluation of high performance boars

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Introduction

The reproductive development of current creation of swine is a major aspect who must to be taken into account when someone wishes to raise the productive indices of farms. Using artificial insemination (AI) in swine farms, a boar has a greater economic impact in regarding reproductive efficiency of the farms if compared to female, because semen of a boar can be used in the AI for a higher number of females (2). An important characteristic to be observed in boars is testosterone production. That hormone is extremely important in male physiology because it stimulates the spermatogenesis, preserves epididymis' sperms (1), stimulates sexual comportment and other secondary characteristics (3). This study's objective has been testing the testosterone levels between the different types, and at the same time, analyzing if testosterone levels in seminal plasma could be an important tool in boar's evaluation.

Materials and Methods

Six high performance DZO-UFLA boars maintained in the same management condition were used in this study. The boars belonged to two different types, been three animals of type A and three other animals of type B, and were, approximately, 20 months old. It was collected eight semen samples from the animals during November 2007 to January 2008. The *in natura* semen was used to perform andrologic examinations (sperm motility, sperm vigor and sperm concentration) and one mL of semen was centrifuged and stored like seminal plasma in -26ºC. After that, the samples were thawed and the testosterone levels were performed using Direct Free Testosterone ELISA Kit. It was used for statistical analysis the SAS (4) program. The test F was used to compare the boars' types, and Spearman correlation test was used to check if testosterone levels had influence in sperm motility, sperm vigor and sperm concentration.

Results and Discussion

Through test F, it can be observed that there was not any difference (P>0,05) between the boars' types in testosterone levels in seminal plasma (Table 1). In Table 2, there were not any correlations between testosterone levels in seminal plasma and sperm motility, sperm vigor and sperm concentration. Therefore, according to the study, it can be concluded that testosterone levels is not a significant tool to evaluate boar's quality.

Table 1. Testosterone levels of different types of high performance boars measured in picogramas per mL.

Types of boars	Testosterone levels in seminal plasma (pg)	Variability coefficient (%)
A	6,10	1,19
B	6,31	

Table 2. Correlation between testosterone levels in seminal plasma and sperm motility, sperm vigor and sperm concentration.

Testosterone levels	Sperm motility	Sperm vigor	Sperm concentration
Seminal plasma	0,00	0,17	-0,02
P =	*0,998*	*0,2539*	*0,9154*

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Morphology of swine semen stored in different extenders and volumes used for intra-uterine artificial insemination

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Introduction

Artificial insemination in swine is an indispensable component for successful management of reproduction. However, the use of this technique still presents some limiting factors such as the use of very high volumes and number of spermatozoa. For this reason the artificial intra-uterine insemination has been much more studied and researched (1). This work aims to evaluate the sperm morphology of swine semen using three different extenders (Androstar, BTS and Merck III – MINITUB of Brazil®) and four volumes (15, 20, 25 and 30 ml) destined to artificial intra-uterine insemination.

Materials and Methods

Three high performance boars were used from the Swine Production Sector of DZO/UFLA and four ejaculate from each animal were collected. The collection was made through hand gloved method and the rich and poor fractions of the semen were collected into a container previously heated at 37°C. After the collection, the ejaculate was fractioned in three similar volumes and each one diluted in the proportion of 1:1 into the following diluents: Androstar, BTS and Merck III. Then, it was determined the concentration of each one of the three fractions and afterwards, of each one was withdrawn four aliquots of semen corresponding to 1.5 billion spermatozoa, and each aliquot put into a plastic flasks and added the respective extender in order to obtain final volumes of 15, 20, 25 and 30 ml. The 12 flasks were kept at 15ºC in fridge and it was made the evaluation of the spermatic morphology of each volume at zero hour (collection day) and 48 hour after it. In the evaluation of the morphology, approximately five semen drops *in natura* were placed into 1 ml formol-citrate solution 2.94% for each of the flasks. After that, an aliquot of this solution was placed between the slide and the coverslip, observed into the phase contrast microscope (magnification 1000 x), and 200 cells were evaluated. The percentage of abnormal heads, tails and the total of alterations were evaluated.

Results and Discussion

The average values of the total morphological alterations are presented in Table 1. No difference (P>0.05) was observed in relation to the morphological alterations of head, tail and total morphological alterations among extenders Androstar, BTS and Merck III in volumes of 15, 20, 25 and 30 ml. The evaluation of semen morphology provides relevant information to determine the quality of semen to be used for artificial intra-uterine insemination technique.

Table 1. Average values of the total morphological alterations observed.

Time (h)	Extenders	15 ml	20 ml	25 ml	30 ml	Average
0	Androstar	8.00	8.67	9.58	8.92	8.79
	BTS	8.17	7.08	7.83	8.50	7.90
	Merck III	6.33	8.42	8.67	7.08	7.63
	Average	7.05	8.06	8.69	8.17	
48	Androstar	7.25	7.00	8.67	8.25	7.79
	BTS	8.17	7.58	9.67	8.33	8.44
	Merck III	9.58	7.92	9.42	9.50	9.11
	Average	8.33	7.50	9.25	8.69	

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Season influence upon seminal plasma proteins in Tabapua breed *Bos taurus indicus*

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Introduction
Seminal plasma is a complex of secretions of the male accessory reproductive organs and appears to exert important effects on sperm function (1). The protein quality of the seminal plasma may affect positively the bulls fertility (2). Peptides of 55 and 66 KDa were present in bulls with excellent spermatic conditions for example motility and vigor. On the other hand, 16 and 36 KDa peptides were observed with unfavourable spermatic conditions (3). The objective of this study was to determine the influence of season upon seminal plasma proteins in Tabapua breed.

Material and Methods
Semen from eleven Tabapua bulls, 30 months old, were collected by electroejaculation during winter (from June to August) and summer (from December to February) of 2007. From each bull a total of 132 semen samples were collected in an interval of 14 days. Samples of seminal plasma were centrifuged (1500g/15min) and conditioned in criotubes and stored at –20ºC until further processing. Proteins were extracted from 200 µL of each sample in 2 mL of extraction buffer composed by 0.625 M Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol and 20% of glycerol. Proteins were quantified according to (4) and electrophoresis was performed according to (5). Gels were fixed with isopropanol: acetic acid: water (4:1:5 v/v) for 30 minutes, and stained in the same solution with 2% of Comassie Blue R250. Percentage of different seasons including plasma proteins were statistically compared by the Chi-square test with significance level at $P<0.05$.

Results and Discussion
In two bulls, the absence of high molecular weight (HMW 55KDa, 66KDa and 80KDa) proteins was verified in the summer. There was a significant increase ($P<0.05$) in total spermatic defects in these two bulls. The protein of 40KDa which suppose to be of low fertility was observed in eight bulls in the summer with semen quality decrease. The eight bulls showed presence of HMW (55KDa) in the winter. In nine bulls HMW (55KDa, 66KDa or 80KDa) proteins were present with a satisfactory semen condition in accordance with (3). The two bulls showed presence of HMW proteins (66KDa and 80KDa) in the summer. The results suggest that different seasons of the year may influence the presence of a variety of proteins in seminal plasma. There was a direct relationship of the season upon seminal plasma proteins. The presence of the proteins of 20KDa, 55KDa, 66KDa and 80KDa suggests an increase of the semen quality during the winter.

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Reproductive suitability evaluation of young bulls selected for yearling weight

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Introduction

A breeding program based on yearling weight performance, with *Bos indicus* breeds (Nelore, Guzerah, and Gir) and the tropically adapted *Bos taurus* breed (Caracu) has been conducted at the Estação Experimental de Zootecnia de Sertãozinho (São Paulo, Brasil) since 1978. The objective of this paper was to access the effect of weight, age and scrotal circumference (SC) on the male reproductive suitability (suitable or not suitable).

Materials and Methods

A total of 192 Nelore, Guzerah and Caracu young bulls, between 21 to 24 months of age, representing the animals classified above the average for yearling weight, within year and breed, were used. The semen was obtained through electro-ejaculation. The animals were classified in suitable =1 or not suitable =0. They were considered suitable when: motility ≥60%, vigor ≥3, major defects <15 and total defects <30 (1). The least-squares means (Table 1) were estimated using GLM and GENMOD procedures (SAS Inst. Inc., Cary, NC), with a model including the breed and year effects and the interaction. The reproductive suitability (SUIT) was analyzed as a binomial distribution trait with a probit link function in a generalized linear threshold model, fitting the effects of weight, age in months and SC class (*B. indicus:* excellent ≥31.5 cm; very good 28.5 < 31.5 cm; good 26 ≤ 28.5; questionable <26cm (2) and *B. taurus:* very good ≥39cm; good 32 < 39cm; questionable ≤32 cm (3)).

Results and Discussion

Caracu differed in age and weight in relation to the others breeds, even though they were contemporary (Table 1). The Caracu SC mean was higher than that related in the sire summary, of 28.2 ± 3.47 cm for yearling animals.

Table 1. Least square means (± standard error) of weight, SC, age and SUIT for Nelore, Guzerah and Caracu young bulls

	Nelore (n=99)	Guzerah (n=44)	Caracu (n=49)
Weight (kg)	464 ± 4.2[a]	449.9 ± 6.3[a]	426.5 ± 6.0[c]
SC (cm)	32.3 ± 0.2[a]	33.0 ± 0.3[ab]	33,8 ± 0.3[b]
Age (month)	22.8 ± 0.1[a]	22.6 ± 0.1[b]	23.0 ± 0.1[c]
SUIT (%)	73.7[a]	72.7[a]	67.5[a]

Different letters in the same line ($p < 0.05$)

The weight, class of SC and age effects were not significant ($p > 0.05$) for the reproductive suitability, however there was significant difference for this trait between 23 and 24 months of age (Figure 1). Studies in Nelore young bulls (4) reported a low correlation between SC and sperm pathologies and it was higher only for bulls up to 18 months of age. Our results showed that SC did not influence the male reproductive suitability between 21 to 24 months of age.

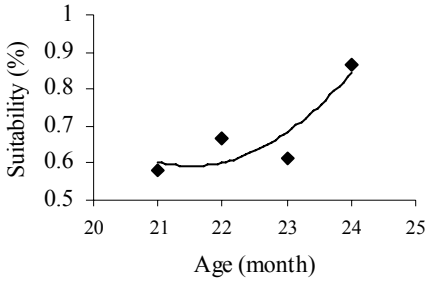

Figure 1. SUIT least square means by age.

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Use of chromomycin A_3 staining in bovine sperm cells for protamine deficiency detection

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Introduction

Commercial bovine embryo transfer has become a large international business. It is well recognized that *in vivo* derived embryos are of superior quality compared to those obtained by *in vitro* maturation, fertilization and culture (1). Pregnancy rates following transfer of *in vitro* produced bovine blastocysts are lower than using *in vivo* produced counterparts (2).Sperm chromatin integrity is essential for the accurate transmission of male genetic information. A condensed chromatin structure is important to maintain this integrity. Protamine (a spermatozoa nuclear protein) is responsible for sperm DNA stability and package during fertilization. DNA damage in bovine sperm does not block fertilization and early embryonic development but induces apoptosis after first cleavages and subsequent embryo death (3). From these results, this work aims to investigate the protamine status in different batches of frozen semen from *Bos indicus* bulls (Nelore).

Materials and Methods

Frozen semen from 14 bulls (4 batches per bull) were thawed and fixed in Carnoy's solution. Smears were prepared and stained with chromomycin A_3 (CMA_3) and fluorescence analysis of the slides was performed. Five hundred sperm cells were evaluated per slide. As positive control of CMA_3 staining, sperm from one random bull was submitted to sperm nuclei deprotamination (4). Evaluation of CMA_3 staining (Figure 1) was carried out by distinguishing spermatozoa with bright yellow staining (CMA_3 positive) from spermatozoa with dull yellow staining (CMA_3 negative).

Results and Discussion

The percentage of CMA_3–positive bovine sperm cells did not vary among batches. Only bulls 1 and 9 showed higher percentage of sperm cells positive for CMA_3 when compared to the others. The means ± SEM of sperm cells positive for CMA_3 from bulls 1 and 9 were, respectively, 0.002 ± 0.0006 and 0.002 ± 0.000 while the means ± SEM from other bulls were not higher than 0.0011 ± 0.0005. The lack of this nuclear protein may occur for many reasons as stress, drugs, smoking, alcohol and life habits (5). Nelore bulls are well adapted to tropical weather and do not suffer with heat stress as other bulls like Holstein bulls (6). As these semen donors were from an Artificial Insemination Center, they were already selected for semen attributes and reproductive performances, explaining the low protamine deficiency. In conclusion, infertility in bulls might not be linked to protamine deficiency per se.

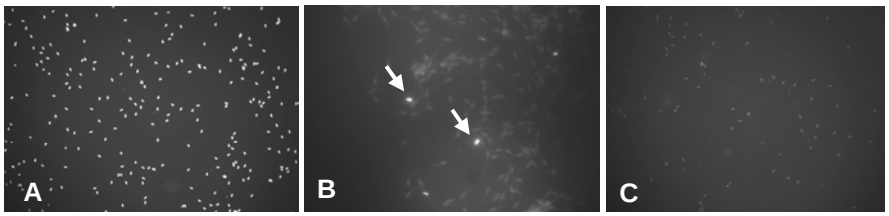

Figure 1. Illustrative images from bovine sperm cells stained with CMA_3. (A) Deprotamination protocol showing all sperm cells deprotaminated. (B) Arrows show positive sperm cells for CMA_3 staining. (C) A sample with no cells stained with CMA_3.

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Cyclophosphamide to prepare mice as an efficient donor for spermatogonial transplantation

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Introduction

The transplant of spermatogonia is an efficient method to study spermatogonial biology. It has been shown that transplantation success depends on the types of cells the donors can provide from their testes cell suspension (1). The best cell suspensions have healthy stem-spermatogonia, in adequate number, and low number of mature germ cells (spermatocytes and spermatids). Despite the previous studies, still there is no general agreement about which model could be the best stem-spermatogonial donor. The purpose of the present study is to determine whether the cyclophosphamide (CY), a chemotherapeutic drug, can cause a temporary depletion of the spermatogenic process becoming a good source of spermatogonia.

Materials and Methods

To determine the adequate dose and via of administration, adult males C57BL6 mice were submitted to the following treatments (n = 3): 1) Single injection of different doses of CY (150 mg or 200 mg/Kg of Body Weight) trough intra peritoneal (i.p.) or per oral (p.o.) via, and sacrificed 28 days after the injection; 2)Multiple doses of CY at 150 mg/Kg (every 5 days, total of 6 injections,via i.p. and p.o.) during 25 days and sacrificed at different end points counted after the first injection (28, 59, 62, 65, 70 and 81 days). The evaluation of the CY effect over the spermatogenesis was determined by sperm head counting (2), and spermatogonial number using high resolution light microscopy (3).

Results and Discussion

After the single administration of CY, (150 and 200 mg/Kg), the sperm head count was similar between the groups (Fig. 1A). However, the animals that received 200 mg became too debilitate, not depending on the type of via used, indicating the dose of 150 mg as the most adequate. In order to eliminate possible surviving cells that would generate late germ cells, it was performed multiple administrations of 150 mg/Kg, resulting in a reduction of the sperm head count immediately after the treatment, with gradual recovery up to 81 days after the last injection (Fig. 1B). Nonetheless, the animals that received p.o. administration of CY presented better recovery than the ones that received i.p. injections. When compared to the control, 28 days after the first injection, the number of differentiating spermatogonia (A_1, A_2, A_3, A_4, In and B) was lower for both treatments (Fig. 1C), but the number of type A undifferentiated (A_{und}) spermatogonia (group which contains the stem-spermatogonia) was close to the control for the p.o. and lower for the i.p. treated animals, suggesting that the stem cell lineage, in both injections, should be preserved. Functional studies using spermatogonial transplantation are being performed intending to determine the efficiency of the stem-spermatogonia, after the use of cyclophosphamide as a germ cell temporary depletion.

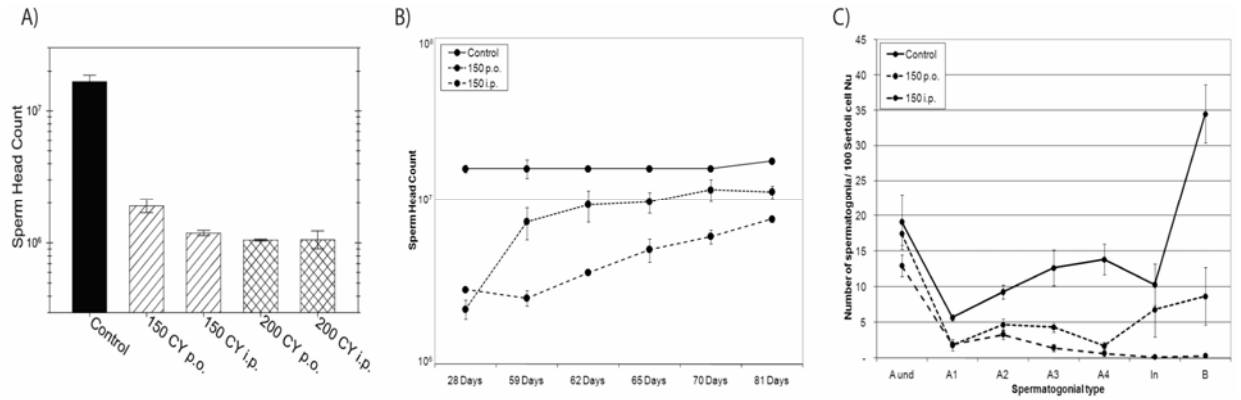

Figure 1. A) Sperm head count from animals treated with one single injection of cyclophosphamide. B) Recovery of sperm head count from animals treated with multiple injections of cyclophosphamide. C) Number of spermatogonia present in animals treated with multiple injections of cyclophosphamide, three days after the last injection. CY-Cyclophosphamide; p.o.- per oral; i.p.-intraperitoneal.

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The human sperm is not necessarily a terminal cell, is probably a indolaminergic cell

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Introduction

Serotonin (5-HT), a neuromodulatory indolamine, is produced in the Central Nervous System and in some cells of peripheral tissues, like the enterochromaffin cells of the gut, the neuroepithelial bodies of the lung, and in the parafollicular cells of the thyroid. Currently, a indolaminergic cell or serotoninergic cell in this case is considered as such when it presents both 5-HT and tryptophan hydroxylase (TPH), -enzyme responsible for the synthesis of 5-HT, in its cell body. In the male reproductive system, both 5-HT and TPH has been described in Leydig cell in testis and epithelial cells in the caput epididymis. In animals, 5-HT is involved in male reproduction; in invertebrates, 5-HT participates directly in sperm motility and fertilization capacities; in mammals, 5-HT promotes the acrosomal reaction. With this in mind, the aim of this work to evaluate if some components of the serotoninergic system are present and are physiologically relevant in human sperm, using both HPLC and immunodetection techniques.

Materials and Methods

Human sperm was obtained from a panel of 6 healthy volunteers with 3-6 days of sexual abstinence. Ejaculates were normal according to the World Health Organization protocol. Sperm cells ($85–120x10^6$) were separated from the seminal plasma using Percoll gradients method. Then, we detect TPH activity in homogenates of both normal and p-chlorophenylalanine (pCPA)-treated sperm during 15 minutes of incubation. Furthermore, we evaluated the direct effects of 5-HT over both sperm motility and protein phosphorylation utilizing CASA system, immunoblotting and densitometric studies.

Results and Discussion

HPLC analyses documented TPH activity in control sperm (n=10, 0.2715±0.052nM/mg protein/hr); which decreases significantly (0.0332 ±0.005nM/mg protein/hr) in the pCPA-treated sperm. In the other hand, 50 and 100 μM 5-HT increases both sperm motility and phosphotyrosine protein levels against control sperm. This data suggests the existence of 5-HT synthesis in the human sperm and gave impetus to growing body of evidence supporting functional role of 5-HT in mammal sperm physiology, suggesting that human sperm is not necessary a terminal cell.

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Fertility and spermatogenesis are altered in male Swiss mice treated with BPP-10c, an angiotensin converting enzyme inhibitor

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Introduction

Recently, we have shown that low molecular weight fraction from *Bothrops jararaca* causes inhibition in the process of spermiogenesis. These data indicated such effects could occur through the BPPs interaction with the tACE, an enzyme expressed in germ cells exclusively during spermatogenesis presenting the C-domain of the sACE with an additional N-terminal sequence (1). The present study was conducted to compare the effect of BPP-10c (<ENWPHPQIPP) and Captopril in the spermatogenesis of male Swiss mice after both molecules administrations.

Materials and Methods

Male Swiss mice (30-35g) were treated with 4.7 μmol/kg for 15 day by i.p of BPP-10c, Captopril or vehicle. Morphological and morphometric parameters of testis were analyzed. The distribution of claudin-1 was done by immunohistochemistry. The BTB integrity after treatment was analyzed by hypertonic fixative method of testes. To examine male anti-fertility and reversibility, male mice treated were housed together with untreated females after 20, 42, 63, 84, 112 and 136 days from the start of administration. All data were presented as the mean ± SEM and the criteria for statistical significance was set at $P < 0.05$ (GraphPad Prism 4.0).

Results and Discussion

We reported that the BBP-10c promoted significant morphology alteration in the seminiferous epithelium, showing an increase of round spermatids number at I, V, VII/VIII stages of spermatogenic cycle when compared with Captopril or vehicle studies. No alteration was shown in the BTB integrity and distribution of Claudin-1 in animals treated with BPP-10c, Captopril and vehicle. We observed that mice treated with BPP-10c were infertile up to day 20. Fertility resumed at day 42 and was fully restored by day 84 when compared with control. The major finding of the present study was the discovery that BPP-10c, modifies the spermatogenesis and fertility in mice. Therefore, these data open new perspectives for the medical development of contraceptive with applications in animals, including humans.

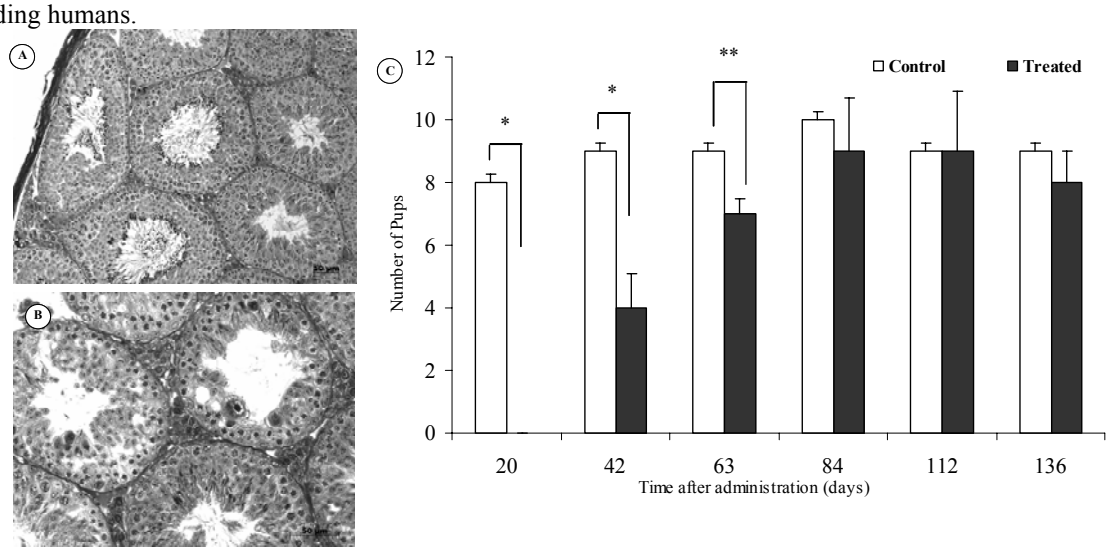

Figure 1. Effect of BBP-10c in the fertility of male adult mice. Morphological analysis in the seminiferous epithelium of male adult mice treated with Captopril (A) and BPP-10c (B); (C) Efficiency of BPP-10c in inducing reversible infertility in adult mice (*$P < 0.001$; ** $P < 0.01$).

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Evaluating testosterone and corticosterone plasma levels in male rats subjected to sperm competition: behavioral and biochemical relationships

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Introduction
Female promiscuity instigates a process of competition among males for the female's reproductive resources. Males, however, have developed physiological responses that allow them to adjust the amount and quality of sperm ejaculated during sexual encounters based upon the magnitude of the promiscuity. This ability reduces the possibility of excessive expenditure of the male's own reproductive resources with females that are highly promiscuous. In spite that prudent sperm allocation has been documented in males throughout the animal kingdom, the physiological mechanisms that underlie it remains obscure. The display of male sexual behavior is controlled, among other factors, by a variety of hormonal and auto/paracrine messengers produced in various organs that constitute the hypothalamus-hypophysis gonads/adrenal axis. It might then be that changes in the plasmatic levels of hormones such as glucocorticoids and androgens are involved in modulating prudent sperm allocation.

Materials and Methods
To begin evaluating this possibility we conducted a correlative study aimed to determine plasmatic levels of testosterone and corticosterone in males subjected to monoandry, biandry or polyandry (four males present during the copulatory session) during sexual encounters. The sexual behavioral performance was carefully recorded and the sperm concentration in the ejaculates determined by directly obtaining semen samples from the uterine horns of the bred receptive female. This procedure was carried out during four copulatory sessions. After the last session all male rats were deeply anesthetized and blood samples withdrawn directly from the caudal cava vein. Blood samples were centrifuged and the serum tested for testosterone and corticosterone concentration through ELISA. Results obtained for sexual behavior, sperm concentration, 5α-DHT and corticosterone were tested with one-way ANOVA followed by Bonferroni post-hoc test. The value of $p<0.05$ was accepted as statistically significant.

Results and Discussion
Our results show that the number of mounts and intromissions, as well as the ejaculatory latency decreased as the magnitude of competition increased. Sperm allocation in the female genital tract was greater in males that competed under a "biandrous" scheme and lower in males subjected to polyandrous copulatory sessions. Surprisingly, no significant differences in testosterone levels were found in blood samples withdrawn from males subjected to different competition paradigms. In contrast, the levels of corticosterone increased as competition did it. Sexual performance had an inverse relationship with corticosterone plasma levels. There was an inverse relationship between testosterone and corticosterone plasma levels. Hence, our results suggest that corticosterone increases as the magnitude of competition among males increases, and that such increments are associated with reduced sexual performance and sperm allocation.

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Acknowledgments
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Seminal plasma proteins and associations with semen traits of beef bulls raised in Northern Australia

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Introduction

Seminal plasma (SP) is a complex mixture of secretions, which include proteins mainly from the cauda epididymis and accessory sex glands. If we mention only events occurring after ejaculation, proteins of the SP affect sperm capacitation and hyperactivation, motility, protection of sperm against protein precipitation, complement-mediated attack from the female immune system, acrosome reaction, interaction of sperm with the oviduct epithelium, zona pellucida binding and penetration, sperm-egg fusion, fertilization and stages of early embryonic development (1). Given these functions, it is reasonable to hypothesize that the expression of some of the seminal plasma proteins influences the fertilizing capacity of the male. The present study was then conducted to determine if seminal plasma proteins are meaningfully related to fertility criteria of beef bulls raised in Northern Australia.

Materials and Methods

Semen was collected from 20 adult *Bos indicus* and composite bulls by electroejaculation, centrifuged at 700 g for 15 min. and the SP was stored in liquid nitrogen. Samples were centrifuged once more at 10,000 g for 60 min. and an aliquot used to determine total protein concentration. Seminal plasma proteins (500 μg/gel) were separated by two-dimensional electrophoresis, gels were stained with Coomassie blue, scanned and analyzed using PDQuest software (Bio Rad, USA),as previously described (1). Protein quantities in the gels were calculated as PPM of the total integrated optical density of the spots. An aliquot from each ejaculate was also used for evaluation of sperm criteria. Statistical differences between animals were evaluated by t test ($p < 0.05$).

Results

Comparisons of SP protein maps from bulls used in the present work and Holstein bulls show a high degree of similarity. However, tropically-adapted Australian bulls have 5 times more albumin in the SP than dairy bulls evaluated in our previous studies. Albumin is multifunctional and its high expression in the SP may be important for adaptation of bulls to conditions of potential heat stress. Bulls with low massal motility (≤3.0; n = 9) had 4.5-fold more of protein spot 101 (4.5-fold) and spots 5901, 6901 and 8802 (5.4, 3.3 and 2.3-fold, respectively) than bulls with high motility (≥3.5; n = 12). The last three spots belong to a train identified previously as clusterin but the identity of spot 101 is still unknown. A regression model with spots 4801 and 4802 as independent variables gave an R^2 of 0.36, showing a quadratic association between their intensity in the SP map and massal motility. In addition, regression analysis including spots 101, 4801 and 8802 gave an R^2 of 0.54 ($p = 0.007$). Spots 4801 and 4802 are isoforms of TIMP-2, a protein also identified as a fertility marker in American beef bulls. Groups of bulls with ≤83 % of normal cells (n = 9) and bulls with more than 83% of normal sperm in the ejaculate (n = 11) differed as regard to the intensity of two clusterin isoforms (spots 8802 and 8805). Sperm morphology is closely related to field fertility of Australian beef bulls (3). Thus, significant associations exist between sperm parameters and SP proteins in tropically-adapted bulls. These preliminary results have set the foundations for a larger study that is being conducted with 75 bulls, with a wide range of fertility indexes.

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Effects calorie restriction on biometric and reproductive parameters of pubertal and fully sexually mature male Wistar rats

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Introduction

The study of the effects of calorie restriction (CR) is receiving growing interest due to its role in prolonging lifespan, which has been established in several species, including some evidences in non-human primates (1). Contrariwise, CR negative impact on reproduction is also reported, though seemingly negligible if very mild (2, 3). Laboratory rodents, especially mice and rats, have been broadly used in CR studies and exhibit extension in lifespan along with an impairment of reproductive function normally proportional to the level of CR applied. The inhibition of gonadal function by CR is a point of debate when envisioning the use of this methodology to prolong lifespan in humans. To investigate the effects of 30% CR on various biometric parameters and reproductive function during post-natal development, male Wistar rats were submitted to calorie restriction from weaning until the ages of 50 and 150 days (d).

Materials and Methods

Sixteen male Wistar rats were subjected to 30% CR starting at age of 24d and ending at ages 50d (n = 8) and 150d (n = 8). As control groups per age, 16 age-matched animals had free access to food (*ad libitum* - AL) and were sacrificed along with CR animals (CRs). Twenty-one-day-old rats were obtained and placed in plastic cages with free access to tap water, 12h light/12h dark and temperature of 22ºC. Animals and food were weighed daily. Thirty percent CR was attained by providing CRs with 70% of food consumed by AL animals (ALs). Starting at day 21, rats were checked daily for balano-preputial separation, to determine onset of puberty. At sacrifice, various organs were collected and weighed: testes, seminal vesicles, prostate + coagulating glands, epididymes, epididymal fat pads, thyroid, adrenals, heart, liver, spleen, kidneys, and pituitary. Testis were routinely processed for histological and histometrical evaluations. A group of males (150d) was submitted to a fertility testing (at 110d). Briefly, female Wistar rats were evaluated for estrous cycle and were bred individually with one male for one night when in proestrus. Females were sacrificed on day 20 of pregnancy, and number of viable fetuses, resorptions and corpora lutea were recorded.

Results and Discussion

In the present study, CR had significant effect on body weight (BW), what is consistent with several other reports. Final BW of CRs was 26 and 20% lower than ALs at ages 50d and 150d, respectively. On the other hand, the onset of puberty and the fertility performance were not affected significantly by diet in the present study, contrasting with some reports in the literature. Absolute weights of most organs were decreased with CR, but indices (organ weight/BW) were affected by CR only in testes and epididymal fat pads. Of interest, and perhaps indicating a short delay in testis development, 30% CR led to reduced seminiferous tubule and lumen diameters in 50d old rats.

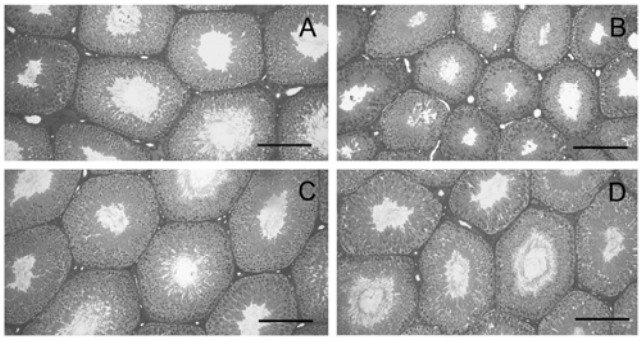

Figure 1. Light microscopy of testis of male Wistar rats. Cross-sections of seminiferous tubules of animals AL 50d (A), CR 50d (B), AL 150d (C), CR 150d (D). Note in 50d old animals the smaller diameter of tubules under CR. Four-µm sections stained with toluidine blue-sodium borate 1%. Bar = 200µm.

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Bilateral cryptorchidism in Morada Nova breed

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Introduction

Cryptorchidism is the no descending of the testicles into the scrotum, and it is more often unilateral than bilateral and this condition is reported in sheep of several breeds (1). Morada Nova is a meat hair breed, originated in the Northeast region of Brazil, with a probably African ascendant (2). The National Association of Sheep Breeders in Brazil considers cryptorchidism an eliminatory defect in rams (3). The occurrence of cases of criptorquidism in a Morada Nova flock in São Paulo State, Brazil, is reported.

Materials and Methods

The flock was formed in 2005, when 23 ewes and two rams were bought. These animals came from three different sheep farms: A) 2 males and 8 females from Franca/SP; B) 7 females from Sales de Oliveira/SP and 7 females from Brasília/DF. This small flock is intensively managed and all the animals were submitted to the same environmental, sanitary and management conditions. Rams were clinically examined and some without testicles inside the scrotum were sacrificed along the time. The reproductive organs of a one-year-old male were taken for histopathological study, and the cariotype was analyzed in another two cryptorquidic males that are still alive.

Results and Discussion

The frequency of bilateral cryptorquidism in the flock was 6.7% (1/15) in 2006, 16.0% (4/25) in 2007 and 6.7% (1/15) in 2008. It was performed a genealogic study of these cases and it was verified that all the animals had an ascendant in common, the same ram, and some of them had the same mother. No cytogenetic abnormalities were observed in the two cryptorquidic males that are alive. In the ram that was sacrificed, the necropsy revealed the presence of both testicles inside the abdominal cavity. The retained testis and epididymis were hypoplastic (Fig. 1A). Histopathological findings (Hematoxilin/Eosin staining) in the testicles revealed seminiferous tubules lined only by degenerated Sertoly cells, and there was no spermatogenesis (Fig. 1B). All the findings lead us to suppose that the cryptorquidism may be associated to a herdable gene that came from their parents, because the flock has a high degree of consanguinity. Further genetic studies should be performed for investigating the role of the male and the female in the transmission of this defect in Morada Nova breed.

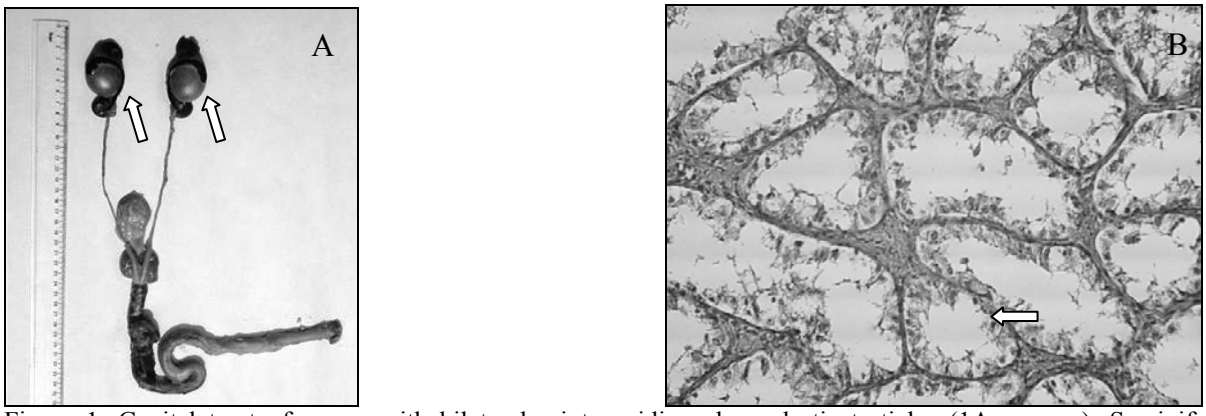

Figure 1. Genital tract of a ram with bilateral criptorquidism: hypoplastic testicles (1A, arrows). Seminiferous tubules lined by degenerated Sertoly cells (1B, arrow).

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Increased sperm morphological abnormalities associated with exposure to urban environmental particulate matter

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Introduction

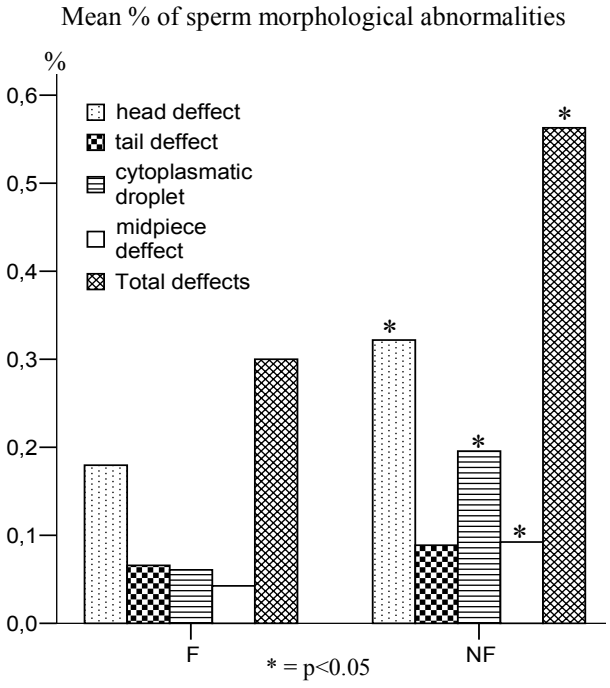
Reproduction can be adversely affected by exposure to a wide variety of environmental pollutants, including poly-chlorinated biphenyls, heavy metals, poly-aromatic hydrocarbons, and particulate air pollution. Sperm morphology is the result of a complex process of cellular modifications occurring during spermatogenesis and morphological abnormalities may be associated with sperm dysfunction and decreased fertility. Recently we observed that couples of mice chronically exposed to particulate matter presented decreased fertility and gestation indices (1). The purpose of this study was to examine the influence of chronic exposure to urban air particulate matter in the city of São Paulo on adult mice sperm morphology.

Materials and Methods

Mice were maintained for two generations (G2) in exposure chambers situated close to a busy urban crossroad in São Paulo. Two groups of G2 mice were raised in normobaric chambers with filtered (F; n = 6) or non-filtered (NF; n =7) air. In NF chambers, three filters eliminated different sizes of particulate matter but not gaseous pollutants. The 24-hr concentration of particulate matter with a diameter of less than 2.5 μm ($PM_{2.5}$) was determined gravimetrically. To examine sperm morphology, 16 week-old male mice were euthanized and sperm was obtained from the cauda epididymides, fixed in Hancock's buffered formol saline solution, placed on glass slides, and observed under phase contrast microscopy under 1000X magnification. A total of 200 sperm per sample were evaluated and sperm head, tail, and midpiece abnormalities and the presence of cytoplasmic droplets were recorded. Statistical comparisons between groups were done using Student's t-test or Mann-Whitney test for independent samples.

Results and Discussion

Concentrations of $PM_{2.5}$ in F (6.5 $\mu g/m^{-3}$) chambers were significantly lower (76%, $P<0.001$) than those in NF chambers (27.5 $\mu g/m^{-3}$). Bodyweight of F mice was 14% ($P< 0.04$) higher than of NF mice. No significant difference in testicular and epididymal weights was observed between the groups. Mean percentages of sperm abnormalities are presented in the graphic (*means $p<0.05$). Our results indicate that the exposure to urban air particulate matter is associated with higher percentage of sperm with morphological abnormalities that could be one of the factors involved in decreased fertility indices observed in mice couple exposed to environmental urban air pollution.



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Morphometric characterization of Santa Ines ram sperm heads using Sperm Class Analyzer® computer-assisted system

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Introduction

The subjective evaluation of sperm morphology has been employed in the diagnosis of male fertility, but large variations between technicians are known to exist which led to the development of computer assisted sperm head morphology analysis. The aims of the present work were to morphometrically characterize Santa Ines ram spermatozoa using Sperm Class Analyzer® (SCA) computer-assisted system.

Materials and Methods

Four Santa Ines adult rams were used for semen collection twice a week from June to July of 2008 (10 collections/animal) by artificial vagina. The ejaculates were evaluated for volume, aspect, sperm concentration and wave motion. Ten microliters of ejaculate was extended in 400 µl citrate-glucose solution (1) at 37°C and 5 µl of this solution was smeared onto slides, air-dried, fixed in glutaraldehyde (3%, pH 7.4), stained with Bengal Rose solution (0.8%, pH 5.3) for 20 minutes, washed several times and after a second air-dried the slides are permanently sealed with a coverslip. At least 150 properly digitized sperm heads were analyzed using the morphological module of SCA. Four morphometric dimensions for length (L), width (W), perimeter (P) (in µm) and area (A) (in μm^2) were assessed. The values of the morphometric parameters were expressed as mean ± standard error and analyzed by ANOVA. If this produced significant F-values, a Tukey HSD test was used for multiple comparisons. Effects were considered significant at $p < 0.05$.

Results and Discussion

The mean of sperm head dimensions of all samples for length, width, perimeter and area were, respectively, 8.22 ± 0.24 µm; 4.51 ± 0.09 µm; 22.05 ± 0.55 µm; 32.10 ± 1.44 μm^2, and are less than those obtained by Monteiro et al. (2007) (2) and Lal and Pant (1982) (3), but superior than those obtained by Gravance et al. (1997) (4) using cryopreserved semen. These results might be due to different staining technique (5) and breed (3). There were significant differences between rams for L, P and A (Table 1), suggesting individual variability (2, 3). However, the low coefficient of variation for all parameters indicates that ram spermatozoa are relatively uniform within individuals. The morphometric analysis of ram spermatozoa using a computer-assisted system is accurate and precise, permitting to analyze a large number of spermatozoa.

Table 1- Mean and coefficient of variation (CV) for morphometrics parameters of individual rams

Animal	Length (µm)	CV(%)	Width (µm)	CV(%)	Perimeter (µm)	CV(%)	Area (μm^2)	CV(%)
A	$8.15 \pm 0.31^{a,b}$	3.80^{a}	4.56 ± 0.18	4.00	$22.00 \pm 0.65^{a,b}$	3.00^{a}	$32.20 \pm 1.70^{a,b}$	5.20^{a}
B	8.33 ± 0.31^{a}	3.70^{a}	4.47 ± 0.17	3.80	$22.17 \pm 0 .67^{a}$	3.00^{a}	$32.20 \pm 1.80^{a,b}$	5.50^{a}
C	8.38 ± 0.32^{a}	3.80^{a}	4.52 ± 0.17	3.80	22.41 ± 0.70^{a}	3.10^{a}	32.90 ± 1.90^{a}	5.60^{a}
D	7.99 ± 0.38^{b}	4.80^{b}	4.46 ± 0.19	4.10	21.53 ± 0.84^{b}	3.90^{b}	30.80 ± 2.10^{b}	6.90^{b}

Means in the same column with different superscripts are significantly different ($p < 0.05$)

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Photoperiodicity and sexual behavior on the isogenic F344 (Fischer) rat

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Introduction

F344 (Fischer), an isogenic rat strain, is known to be more difficult to breed than other strains. Interestingly enough, even being the F344 the most commonly used isogenic rat strain, little is known why its breeding is still a challenge. Shoemaker et al. (2002) found that photoperiodicity affects the F344 reproductive development, considering that testis and seminal vesicle size, puberty and development growth can be affected by the standard laboratory photoperiod of 12 hours. Although several studies versed on F344 photoperiodicity and sexual development, none verses on how different photoperiods affect the sexual behavior on this rat strain. The current study analyzes the sexual behavior of F344 females under the standard laboratory photoperiod and longer (than 12h) photoperiods (14h and 16h) to determine if, and in what degree, the females of this strain are sexually photo responsive and if the standard photoperiod is suitable to F344 breeding programs.

Materials and Methods

The animals were obtained from the SPF breeding facility of the IQ-USP. Twenty-seven females were caged in groups of three and submitted to three different light/day (l/d) cycles. Males were placed together with the females and allowed to stay overnight. Next morning, vaginal washes were used to determine the oestrus period while the presence of vaginal plugs and spermatozoa were indicative of mating. Group I was in the standard 12/12h l/d cycle schedule, Group II was in photoperiod longer than 12h, split in two: subgroup II.a (14/10h cycle) and subgroup II.b (16/8h). All the males were in a 12 l/d cycle.

Results and Discussion

Group I - standard 12h photoperiod - had a 5.6% mating rate. Group II - longer than 12h photoperiod - had a 13.8% mating rate (II.a – 12.9%, II.b – 14.8%), suggesting that the F344 females were sexually responsive to an increasing photoperiod. [Fig.1]. The Mann-Whitney test resulted in a p-value of 0.0461 between Groups I & II (at confidence level 95), indicating that results are statistically significant. The results indicate, for the first time, that the F344 should be considered a photoperiodic rat strain, sexual behavior concerned. This is an ongoing study, and there is still a need to address how different light/dark cycles would affect the males` sexual behavior on this strain, and what light/dark schedule on males/females of F344 should be considered better for breeding purposes. F344 breeders and researchers, who work with this strain, should keep the photoperiodicity factor in mind to optimize the breeding management. This characteristic also qualifies the F344 as a possible model for studies on photoperiodicity.

- **Fig.1 - Mating Results**

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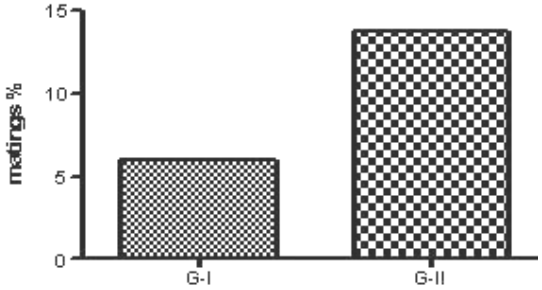

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Paraprostatic cysts in orchiectomized dogs: a case report

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Introduction

Prostatic diseases are common in large-breed older male dogs. As age advances, a gradual increase in the prostate occurs as a result of acinar hyperplasia caused by excessive androgens and/or fibromuscular hyperplasia due to excessive estrogens, leading to prostatopathies, such as benign prostatic hyperplasia (58% of cases), bacterial prostatitis (18.6%), squamous metaplasia, cysts, prostatic and paraprostatic abscesses (5%) and neoplasias (2). The etiology is uncertain, and congenital origin is suggested, but the identifications of embryonic evidence has not been elucidated. Ductal occlusion results in progressive secretory stasis and excessive secretion by glandular tissue, in face of functional duct obstruction, causes fluid retention with cyst formation (3). A common complication is secondary contamination resulting in signs of gland abscessation originated from ascending infection or by the hematogenous route (1). The surgical techniques described for abscesses, prostatic or paraprostatic cysts associated with bilateral orchiectomy are marsupialization, local drainage and/or resection with omentalization, subtotal prostatectomy with capsulectomy or subtotal intracapsular prostatectomy, total prostatectomy and ultrasonography-guided percutaneous drainage (1). This study aimed at reporting the case of an orchiectomized dog which has been affected by a paraprostatic cyst.

Case report

A 12-year-old male Weimaraner dog, orchiectomized for 2 years and with a history of dysuria, oliguria, hematuria, dyschesia and tenesmus, was attended to at the Veterinary Hospital of FCAV/UNESP. The absence of sensitivity to rectal palpation was observed during clinical examination. Hematologic tests resulted in normocytic normochromic anemia; biochemical tests showed normal values and urinalysis presented low urinary density, hematuria and urinary sediment with a large number of red cells, leukocytes, transitional epithelial cells and bacteria. Ultrasonographic examination enabled the visualization of a hypoechoic structure with 15 cm in diameter, floating hyperechoic sediments and a thin hyperechogenic capsule in the craniodorsal position to the bladder and connected to the prostatic parenchyma in the craniodorsal region of the left lobe by a duct with 5 cm in length and 0.7 cm in diameter, thus compressing the colon and rectum. Prostatic parenchyma showed homogeneous echogenicity and a volume of 4 cm. The prostatic urethra was preserved. Regular radiographic examination of the abdomen confirmed the abovementioned alterations, and excretory urethrocystography with positive contrast made it possible to delimit the visualized structure on the ultrasound, thus confirming the paraprostatic cyst diagnosis. The animal was submitted to exploratory laparotomy, resection of the paraprostatic cyst and duct omentalization. A sample of the cyst's capsule was send for histopathological analysis and its content was used for culture and antibiogram. The histopathological examination resulted in chronic prostatitis with cicatricial areas characterized by large necrotic areas, cholesterol clefts, hemorrhage, hematoidin and granulation tissue with cicatrization areas. The culture and antibiogram test were negative. The animal presently shows good overall conditions and has been periodically submitted to clinical and laboratory follow-up.

Discussion

Prostatic diseases are common in large-breed older male dogs, such as the animal in question. As age advances, a gradual increase in prostatic volume occurs due to hormonal action, leading to the gland's hyperplasia and triggering prostatopathies. The patient had been orchiectomized two years before, but had not been investigated in relation to the existence of any existing prostatopathies. It is suggested that the cyst was in development when the animal was submitted to orchiectomy, since this surgery results in a 70% decrease in prostate volume, reducing the possibility of ductal occlusion, secretory stasis and fluid retention (3). The prostate of the animal in question showed a normal volume, thus confirming the efficacy of orchiectomy in relation to hormonal influence on the gland's parenchyma. The treatment of retention cysts or paraprostatic cysts consists in surgical removal. In this case, cyst resection and duct omentalization were chosen.

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Urine and pH effects on canine spermatozoa

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Introduction

During the semen dog collection, the ejaculate can go to the bladder direction, this occurs in pathologic situation or in semen collection by electroejaculation. Previous studies recorded the effects of urine regarding spermatozoa and the high osmolarity and differences between pH of the semen and urine had been responsible for the motility losses in horse (1), human (2) and dog (3). The aim of this study was to compare the effects of urine and effects of acid and basic NaCl solutions.

Materials and Methods

Five ejaculated and autologous urine for different dogs were utilized in this study. The semen collection was performed by digital stimuli of the penis and the urine was collected after spontaneous micturion of the dogs. Aliquots 100 microliters of second fraction of semen were mixed with 900 microliters of seminal plasma, urine or five pH values (5.0; 6.0; 7.0; 8.0; and 9.0) of saline solution of NaCl (150mM), plus HCl (0.1N) or NaOH (0.1N) for achieve desired pH. The effects of these solutions were compared after 1 hour of incubation in water bath at 37°C. The end points evaluated were sperm motility, with *computer*-assisted semen analysis, plasmatic and acrossomal membrane integrity, with eosin yellow and PSA-FITC, respectively. Statistical analyses used Tukey to compare the effects of the urine and different pH values on the spermatozoa in the different columns and Student t-test to compare the pH values found in the semen and the urine. Both analyses were performed in the the Sisvar® software.

Results and Discussion

pH of semen and urine were 6.5 and 6.9; respectively ($p > 0.05$). The effects of all solutions with different pH values and seminal plasma were different from all those found for urine incubation on spermatozoa functionality (Table 1).

Table 1. Results after semen incubation in urine and solutions of NaCl (150mM) with different values of pH.

Variables \ Treatments	SP	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0	Urine
TOTMOT (%)	62.2 ± 35.9^{a}	44.6 ± 40.9^{a}	53.6 ± 30.7^{a}	63.4 ± 21.0^{a}	46.6 ± 25.7^{a}	33.4 ± 35.8^{a}	0^{b}
PROGMOT (%)	32.4 ± 23.5^{a}	$16.2 \pm 14.8^{a,b}$	$7.2 \pm 6.7^{a,b}$	$26.0 \pm 7.9^{a,b}$	$14.4 \pm 17.3^{a,b}$	$16.0 \pm 17.2^{a,b}$	0^{b}
PMI (%)	93 ± 12^{a}	45 ± 11^{b}	42 ± 24^{b}	51 ± 28^{b}	50 ± 13^{b}	$60 \pm 23^{a,b}$	0^{c}
AMI (%)	96 ± 5^{a}	95 ± 2^{a}	92 ± 7^{a}	88 ± 4^{a}	92 ± 10^{a}	96 ± 1^{a}	31 ± 26^{b}

Legend: TOTMOT=Total motility; PROGMOT=Progressive motility; PMI=Plasmatic membrane integrity; AMI=acrossomal membrane integrity; SP=seminal plasma (control).

We observed that the effects caused by urine were greater than that caused by all other NaCl solutions. The effects caused by basic solution were apparently greater than acid solutions in other species (1,2), but in this study these differences were not found. In conclusion, the effects of urine on the spermatozoa functionality were not caused by urine pH. These results will contribute for diluents development for purposes of using the semen contaminated by urine, because these diluents can be used before the contact of the semen with urine or can revert those effects when the contamination cannot be avoided.

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Assessing the quality of the tilapia gametes (*Orechromis niloticus*) in the period of winter, through the hormone induction using GnRH

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Introduction

In most fish farm, the reproductive induction is made through the use of carp pituitary extract, considered standard technical for reproductive induction due to ease of implementation (1). Other substances have been investigated as alternatives in the induction of reproduction in fish. The GnRH acts indirectly in the gonads, inducing the inherent production of gonadotropins in the pituitary. The objective of this study is to evaluate the oocytes and sperm quality of tilapia (*Orechromis niloticus*) in the period of winter, through a hormonal induction using GnRH.

Materials and Methods

The experiment was conducted in the Fish Station of the University Federal of Lavras, in July 2008. We used eight males and 24 females of tilapia, with an average weight of 800g. The animals were separated by family (one male to three females), being two families per cage in a tank of 8x5x1.6 m covered by glass. After adaptation period of 15 days, two families received intramuscular dose of GnRH (25mg/kg body weight) and two families received saline solution. After 12 hours of hormone induction the animals were massaged for collection of oocytes and sperm. We evaluated the rate and duration of the sperm motility, using sodium bicarbonate (0.5%) as activator solution. A sample of 10 µl of the sperm of each animal was fixed in citrate formalin solution to determine the sperm concentration. The diameter of oocytes for each female was measuring in optical microscope with the help of an eyepiece millimeter ruler.

Results and Discussion

Semen volume of 0.5 ml was obtained from each male; two males from the control group and one male from the hormone treatment group. The evaluated semen showed no significant difference ($p > 0.05$) in the motility rate and duration. The average motility rate was 40% and 9 minutes and 58 seconds for motility duration. The average sperm concentration was 1.83 $x10^9$spz/ml. The diameter of oocytes of the control (1,17 ± 0,21) and treated fish with GnRH (1,16 ± 0,28) showed no significant difference ($p > 0.05$, Fig. 1).

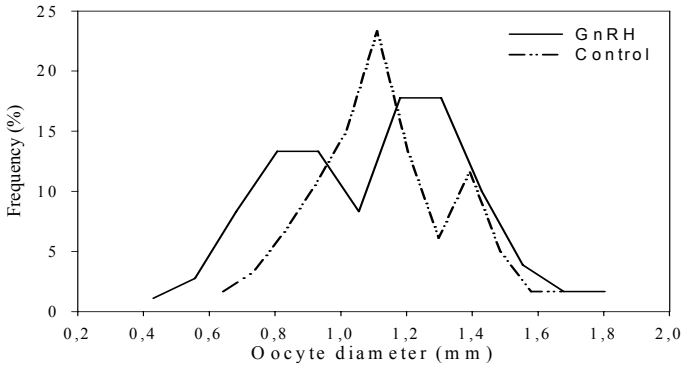

Figure 1. Frequency of oocytes diameter for control and GnRH animals.

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Effect of testicular incisional biopsy on the volumetric proportion of the components of the testicular parenchyma and tubular parameters for dogs and cats

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Introduction

The incisional testicular biopsy is extremely useful to evaluate spermatogenesis in animals and human with fertility disorders, and to describe qualitative and quantitative histological parameters of testicular parenchyma in wild species. This technique has been used with caution due to the lack of knowledge around the effects of the procedure on the testicular parenchyma and how it can affect sperm fertility by damaging the blood-testis barrier. This study aimed to investigate the effects of unilateral testicular biopsy on the spermatogenic process of dogs and cats after 60 days.

Material and Methods

Six adult dogs and six adult cats were used. The animals were submitted to unilateral incisional testicular biopsy with a 4.0 mm diameter skin puncher and orchietomized 60 days after. The testis fragments were immediately immersed into a 4% glutaraldehyde fixative solution and included into glycol metacrylate resin. Then, four slices sectioned at 4.0 mm thick were obtained in a rotary microtome provided with a glass knife. The slides were evaluated on light microscopy to determine: volumetric proportion of seminiferous tubules and connective tissue on the testicular parenchyma, seminiferous tubules diameter and length per gram of testis, Sertoli cell index per total germ cells and the general spermatogenesis efficiency.

Results and Discussion

Adhesion and fibrous tissue were observed at the site of biopsy 60 days after, apparently due to the process of healing. Also the percentage of connective tissue along the testicular parenchyma was increased in the biopsied testes of cats after 60 days. There was no influence of testicular biopsy on the other parameters measured, as described in Table 1. Although invasive procedures in the testes can cause temporary physiological changes, such as production of anti-sperm antibodies (1), incisional testicular biopsy produced no negative long term effects on testicular function of dogs and cats.

Table 1. Diameter of seminiferous tubules (μm), volumetric proportion (%) of seminiferous tubules and connective tissue on the testicular parenchyma, general spermatogenesis efficiency and Sertoli cell index per total germ cells on the biopsy specimen (B), biopsied testis (BT) and contralateral testis (CT), and seminiferous tubules length per gram of testis (m/g) on the biopsied and contralateral testis, 60 days after.

	Seminiferous tubules length per gram of testis	Diameter of seminiferous tubules	Volumetric proportion of seminiferous tubules	Volumetric proportion of connective tissue	General spermatogenesis efficiency	Sertoli cell index per total germ cells
			Dogs			
B	--------	227.1 ± 19.2	83.3 ± 3.2	12.9 ± 3.0	55.9 ± 10.1	13.3 ± 1.9
CT	19.5 ± 0.3	221.3 ± 11.5	83.7 ± 1.6	10.9 ± 1.6	65.3 ± 15.5	16.8 ± 3.5
BT	19.1 ± 1.0	218.6 ± 6.7	84.2 ± 3.0	12.0 ± 3.0	51.0 ± 12.0	14.9 ± 3.7
			Cats			
B	25.2 ± 4.9	189.6 ± 17.1	81.8 ± 3.3	8.7 ± 1.9[a]	75.7 ± 33.6	15.8 ± 4.1
CT	24.0 ± 1.7	188.1 ± 5.0	80.3 ± 5.1	10.7 ± 3.5[a]	68.5 ± 32.5	15.2 ± 3.1
BT	25.2 ± 3.2	185.3 ± 13.2	77.5 ± 3.2	12.5 ± 1.5[b]	75.8 ± 30.8	16.1 ± 2.9

*Values followed by different letters in the same column are significantly different ($p < 0.05$).

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Sperm morphology of a Teleost Fish Tambaqui, *Colossoma macropomum* (Cuvier, 1818)

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Introduction

The tambaqui is one of the Brazilian reophilic fish species of high economic value for the Amazonian region and the Northeast region of Brazil. Little is known about sperm morphology of Brazilian species, or the effects of maturational hormones used to induce spermiation on sperm morphology. Morphology analysis should be included on the evaluation of fresh semen, especially prior to and after freezing. The aim of this work was to analyze sperm morphology of Tambaqui injected with carp pituitary extract (cPE) as spermiation inducer and compare with untreated fish. Sperm morphology was evaluated by a computer-assisted sperm analyzer software (CASA).

Materials and Methods

Twenty six Tambaquis were divided in two groups: one group received a single injection of cPE (2 mg/kg BW) and the other one served as control (untreated). Semen was hand-stripped 12 h after injection and was diluted 1:4000 in a 1% formol saline solution. Then, 100 µL of diluted semen was stained with 2 µL of bromofenol blue dye. The smear was placed in light microscope coupled to a video camera and analyzed with a CASA software (SCA, Micropticals S.L, version 5, Spain). The following sperm morphology parameters were evaluated: normal sperm, head defects, microcephalic head, macrocephalic head, midpiece defects, proximal droplet, distal droplet, coiled tail and bent tail.

Results and Discussion

The results of sperm morphology analysis are depicted on Table 1. Distal droplet and coiled tail were the only defects that were higher in untreated fish compared to cPE-treated fish. All the values observed, however, are within the range accepted by the Brazilian College of Animal Reproduction (1). The use of cPE as spermiation inducer for Tambaqui didn't cause abnormalities on sperm morphology and thus it can be used in fish farms as a routine method prior semen collection.

Table 1. Sperm morphology (%; mean ± SEM) of Tambaqui treated or not with carp pituitary extract.

Defects	cPE-treated fish	Untreated fish
Normal sperm	86.5 ± 8.4	83.1 ± 6.1
Head defect	0.7 ± 0.9	1.4 ± 1.5
Macrocephalic head	0	0.1 ± 0.5
Microcephalic head	0	0
Midpiece defect	0.8 ± 0.8	0.9 ± 1.9
Proximal droplet	0.5 ± 0.7	0.6 ± 1.0
Distal droplet	1.4 ± 1.5	2.8 ± 3.0*
Coiled tail	0.1 ± 0.3	0.6 ± 1.0*
Bent tail	10.0 ± 8.2	10.5 ± 4.1

* Means within a row followed by this symbol are significantly higher ($p<0.05$).

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Cell proliferation and apoptosis during spermatogenesis of *Prochilodus argenteus* in the São Francisco River, downstream from the Três Marias dam, Brazil

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Introduction

Spermatogenesis requires the spatial and temporal coordination of germ cells maturation to ensure optimal production of spermatozoa (1). Programmed cell death for apoptosis plays a major role in the testicular homeostasis. In teleost fish, few studies correlated proportions of cells germ with activities of cell proliferation and death during spermatogenic cycle (1). Previous study in the São Francisco River, downstream from the Três Marias dam, has reported the negative influence of the hydroelectric impoundment on the reproductive activity of curimatã-pacu *P. argenteus* (2). The present study analyzed quantitatively cell proliferation and death during reproductive activity of *P. argenteus* males in two sections of the São Francisco River: first section immediately bellow Três Marias dam (JB) and second section after the confluence with the Abaeté River (JA), where environmental conditions are favorable for breeding.

Materials and Methods

For morphometric analyses 15 mature males of P. argenteus from each river stretch, captured from December/2005 to February/2006, were used. Testis samples were fixed in Bouin's fluid, included in paraffin and processed for histological and immunohistochemical analyses. Tubular diameter and number cysts per seminiferous tubules were measured by digital images obtained in non-sequential histological sections from each specimen. The volumetric density of testicular structures was determined using a 441-intersection grid coupled to the ocular of the light microscope. Fifteen fields chosen randomly were scored for each animal at 400X magnification. The cell proliferation and death were analyzed through immunohistochemistry using PCNA (proliferanting-cell nuclear antigen) and TUNEL *in situ* assay respectively, in five mature testis from each river stretch. The labeled cells were quantified by area of 100 mm^2 at 400X magnification.

Results and Discussion

Morphometric analysis revealed higher tubular diameter, cysts per seminiferous tubules and volume density of spermatocytes and Leydig cells in JA indicating that reproductive fitness of *P. argenteus* males is improved in this river stretch. Cysts and volume density of spermatogonia predominated in JB, where the lower temperature of the water induces a spermatogenesis delay. The PCNA labeling occurred in Sertoli cells, spermatogonia and spermatocytes, as also evidenced in other teleosts (3). Morphometry for PCNA reactions showed higher cell proliferation in spermatogonia and Sertoli cells in JB while spermatocytes and Leydig cells were labeled predominantly in JA (Fig. 1). The apoptosis occurred in all phases of spermatogenesis similar to other species (1, 4). Immediately downstream from the dam, TUNEL reaction (number of labeled cells/area) occurred mainly during espermatogonial phase while in JA it predominated in spermatocytes. These results suggested that morphometric analysis associated with markers such as PCNA and apoptosis can be important parameters for monitoring the spermatogenic activity in teleosts.

Figure 1: PCNA and TUNEL reactions in mature testis of *P. argenteus.* G1= primary spermatogonia; G2= secondary spermatogonia; C1= primary spermatocytes; C2= secondary spermatocytes; S = Sertoli cells; (*) differences between stretches are significant, $P < 0.05$.

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Duration of spermatogenesis and daily sperm production in the ocelot (*Leopardus pardalis*)

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Introduction

Similar to most wild felids, the ocelot (*Leopardus pardalis*) is an endangered species that is currently threatened by habitat loss, fragmentation, and human persecution. To worsen this situation, the knowledge of reproductive biology in the ocelot is very limited. The germ cell transplantation is a fascinating and powerful technique that has been primarily used in the past decade for investigating spermatogenesis and stem cell biology in mammals, particularly rodents. Ongoing studies in our laboratory are showing that the domestic cat can be used as a recipient model to preserve and propagate male germplasm from threatened wild species, including the ocelot. In this kind of investigation, the knowledge of the duration of spermatogenesis and the characterization of germ cells morphology are very important parameters, in order to track the development of transplanted spermatogonial stem cells, after they are transplanted into the recipient testis. In this regard, our main goal in the present study was to investigate the basic aspects related to the testis structure, particularly spermatogenesis, in the sexually mature ocelots.

Material and Methods

Four adult animals weighing approximately 14 kg were utilized. In order to determine the duration of spermatogenesis, intratesticular injections of tritiated thymidine were performed and the animals were orchiectomized at specific time periods after injection. After orchiectomy, the testes were fixed by immersion in 4% buffered glutaraldehyde and the obtained testis fragments were embedded in glycol methacrylate, stained with toluidine blue, and routinely prepared for histological and morphometrical evaluation.

Results and Discussion

As shown in Table 1, the mean testis weight and the gonadosomatic index (GSI) was 11 ± 1 g and 0.16 ± 0.01%, respectively, whereas the seminiferous tubules and the Leydig cells volume density (%) were ~83±2 and ~10 ± 2%. According to the tubular morphology system, eight stages of spermatogenesis were characterized. Each spermatogenic cycle and the entire spermatogenic process (based on 4.5 cycles) lasted approximately 105 ± 0.3 and 47.3 ± 1.4 days in ocelots. Leydig cell individual volume was 2,500μm^3, whereas the number of Leydig and Sertoli cells per gram of testis was 38 ± 5 x 10^6 and 46 ± 3 x 10^6. Based on the number of round spermatids per pachytene spermatocyte (2.9 ± 0.1; meiotic index); significant cell loss (~30%) occurred during the two meiotic divisions. Approximately 4.5 spermatids were found per each Sertoli cell (Sertoli cell efficiency), whereas the daily sperm production per gram of testis (spermatogenic efficiency) was ~20 ± 1 x 10^6. In comparison to the data obtained in our laboratory for the domestic cat (Table 1), the GSI in the ocelot was more than 100% higher. As most of the other parameters are relatively similar for both felid species, we can suggest that the ocelot invest more in sperm production, indicating that these two species might have different reproductive strategies. Finally, we are currently evaluating the progression of ocelot's germ cells transplanted into the domestic cat testes and we hope that they eventually will form donor spermatozoa.

Table 1. Comparative parameters related to the testis morphometry and spermatogenic events in sexually mature ocelots and domestic cats.

Parameters	Ocelot	Domestic cat
Body weight (kg)	13.75 ± 1	3.1 ± 0.2
Testis weight (g)	11 ± 0.6	1.17 ± 0.07
Gonadosomatic index (%)	0.16 ± 0.01	0,078 ± 0.007
Testis parenchyma volume density		
Seminiferous tubule	83.1 ± 1.6	88.2 ± 1.2
Leydig cell	9.8 ± 1.5	6 ± 0.6
Leydig cell volume (μm^3)	2,500 ± 170	2,044 ± 150
Leydig cell number per gram of testis (million)	38 ± 5	30 ± 2.9
Sertoli cell number per gram of testis (million)	46 ± 3	32 ±3.9
Round spermatids per Sertoli cell	45 ± 0.09	5.1 ± 0.6
Spermatogenic cycle length (days)	10.5 ± 0.3	10.4 ± 0.03
Spermatogenic efficiency (million)	19.8 ± 1	15.7 ± 1.6

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Histomorphometry of rats testis after chronic maternal Protein deficiency

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Introduction

Reproduction in mammals is a complicated process that must occur in harmony with existing dietary, physical and social conditions. It has been demonstrated that the availability of dietary protein is a limiting factor for full somatic growth, and for the efficiency of the reproductive process (1). Dietary peptides and amino acids have the potential to modulate reproductive status by acting directly as neurotransmitters or indirectly as precursors to the central nervous system neurotransmitters. They also stimulate a variety of metabolic changes, which provide signals to the reproductive axis (e.g. growth hormone, prolactin, GnRH). Moreover, protein deficiency affects the maturation and growth of the reproductive organs as well as their morphology in animals after birth (1,2). However, few studies evaluated the effects of protein malnutrition from conception up to adulthood. Thus, the aim of this preliminary study was to evaluate the effects of pre- and post natal protein deficiency in some parameters of the testis of rats.

Materials and Methods

To determine the effects of protein malnutrition on the morphology of testis, three groups of pups were established after weaning: (i) control (n=2): male pups that received a commercial diet (containing ~20% crude protein) after weaning and whose mothers also received a commercial diet before breeding, during pregnancy and lactation; (ii) deficient (n=1): male pup that received a protein deficient diet (containing ~6% crude protein) after weaning and whose mother also received the same diet before breeding, during pregnancy and lactation; (iii) returned control (n=2): male pups that received a commercial diet after weaning and whose mothers received a deficient diet before breeding, during pregnancy and lactation. At 70 days of age, the rats were perfused-fixed with glutaraldehyde and testis fragments were embedded in glycol methacrylate (3). Biometrical (body and testes weights), histological and morphometrical (tubular diameter and height of the seminiferous epithelium) data were obtained.

Results and Conclusions

The deficient rat showed low body weight (15.6g) compared to the control (218±32g) and returned control (179±12g) rats. In the same manner, testicular weights were reduced only in the deficient (0.20±0.003g) compared to the control (1.48±0.02g) and returned control (0.93±0.03g) animals. Testicular descent did not occur in the deficient rat and a gross histological analysis of the testes indicated that the seminiferous tubules were adversely affected. For instance, at 70 days of age, the Sertoli cells still presented immature morphology and the most advanced germ cells observed in the seminiferous epithelium were pachytene spermatocytes. On the other hand, the control and returned control animals showed similar normal testicular parameters. The diameter of the seminiferous tubules, the lumen area and the height of the seminiferous epithelium were clearly smaller in the deficient rat compared to the other experimental groups (Fig. 1 and 2). The results of the current study showed that protein deficiency in the pre- and post-natal periods may play an important role in testicular development, severely affecting the cellular constitution of the seminiferous epithelium in adult. Even though the number of animals used was low in this preliminary study, the histological modifications were evident as observed in Figure 2. Further investigations are currently being developed to confirm these findings.

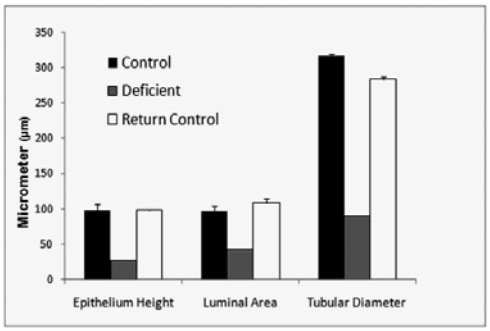

Fig. 1. Comparasion of three morphometrical parameter on the rat testis after different nutritional diet (mean ± sem).

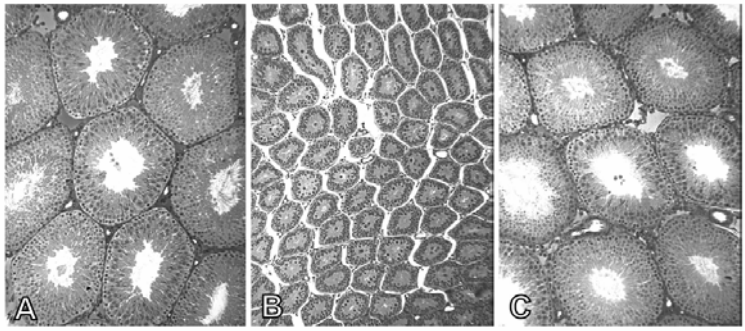

Fig. 2. Photomicrographies of rat testes showing clearly difference between de diameter of the seminiferous tubules of the deficient rats (B) compared with the diameter of those seminiferous tubules of control (A) and returned control (C) rats.

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Development of germ cell transplantation in zebrafish (*Danio rerio*): a means to study the spermatogonial stem cells in fish

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Introduction

In all vertebrates, ranging from fish to mammals, spermatogenesis is dependent on the continuous activity of spermatogonial stem cells (SSCs). SSCs lie at the foundation of spermatogenesis and are the only stem cells in the body that undergo self-renewal throughout life and differentiate to produce a large number of spermatozoa. Knowledge about these cells is generated mainly with transplantation assays that also have great potential applied value, such as for instance, for genetic manipulation, or for conservation of valuable and endangered species. Recently, a germ cell transplantation has been adopted to adult fish successfully in our laboratory using Nile tilapia, and created a new scenario to study SSCs in fish. Zebrafish has become a suitable vertebrate model for basic and biomedical research due to the unique combination of being simple to maintain and house, and also being suitable for genetic and cell biological studies. The aim of this study is to develop the germ cell transplantation technique using this excellent vertebrate model.

Material and Methods

1) Preparation of recipients for germ cell transplantation: To achieve the effective method to suppress the endogenous spermatogenesis, we have tested two concentrations (30mg/Kg and 40mg/Kg per body weight (BW)) of a cytostatic drug, busulfan (Bu), combined with high water temperature (35^{o}C). The animals received a single intra-coelomatic dose of Bu and their testes were examined (histology and cyst frequency) at 2, 4, 6, 8, 10, and 12 days after treatment.

2) Preparation of the cell suspension: Twenty-four males were used as donors. Their testes were digested enzymatically and the obtained cell suspension was submitted to Percoll density gradient centrifugation. The selected (spermatogonial) cell fraction was labeled with PKH26 (Red Fluorescent Cell Linker).

3) Transplantation path: Approximately 10^5 donor germ cells were transplanted into the testes of each depleted recipient. The transplantation path was assessed through the genital pore using a stereomicroscope and a micropippete attached to a micromanipulator.

4) Analysis of the recipient testes: In order to detect and track the transplanted cells, the recipient testes were analyzed under fluorescent microscope at 2, 4, 6, and 8 days after transplantation.

Results and Discussion

1. The recipients' endogenous spermatogenesis was successfully suppressed at 10 days after the dose of 40mg/Kg/BW of Bu (Fig. 1). The high frequency (88%) of *Sertoli cell only* tubules provides ample niches for transplanted donor germ cells.

2. Labeled germ cells are found in the recipients' testes for at least 8 days post-transplantation as shown by PKH26 label. Donor germ cells colonize the testis and proliferate forming spermatogenic cysts in the recipient's seminiferous epithelium (Fig. 2), indicating that germ cell transplantation is feasible in adult zebrafish. Hence, we have taken an important step for establishing the transplantation assay as a mean to study the regulation of SSCs in zebrafish.

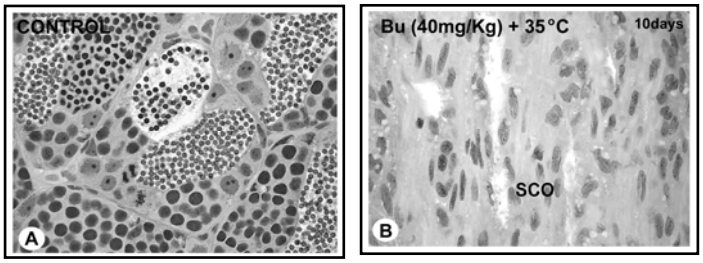

Figure 1. Normal (**A**) and Bu-depleted (**B**) spermatogenesis, where the seminiferous epithelium is composed mainly of Sertoli cells only (**SCO**).

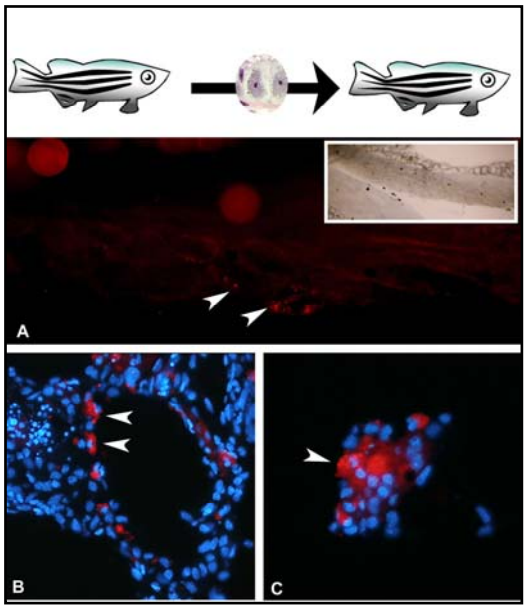

Figure 2. Germ cell transplantation in zebrafish. Labeled donor germ cells (**arrowheads in A**) are found in the recipients' testes for at least 8 days post-transplantation as shown by *in toto* observation of the testis. In **B** and **C**, **arrowheads** indicate that transplanted donor germ cells colonize, proliferate and form cysts.

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Semen Evaluation by Biospeckle Laser and Computer-Assisted Semen Analysis (CASA)

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Introduction

Computer assisted semen analysis (CASA) is currently the most accepted method for assessment of motility (M) and spermatozoa physical characteristics, giving also information related to the cell trajectory, path velocity and movement proportion of mobile gametes, being able to eliminate the intrinsic subjectivity of human evaluation [1]. Biospeckle laser (BSL) is a method of laser artificial vision that generates an index called inertial moment (IM). BSL is a phenomenon related to the dispersion of laser light on the surface of a material showing biological activity. The aim of this work was to compare spermatic kinetics analysis of post-thawing bovine semen using CASA and BSL [2].

Material and Methods

Semen of six sexually mature bulls filled in 0.5 ml straws with concentration adjusted to 30-35 million cells per straw were thawed at 37 ° C for 30 seconds in a water-bath. In the BSL essay, the inertial moment (IM) was generated, an index that quantifies the activity of biological materials. Sixty semen samples (10 of each bull) were used. The experimental set up in the BSL was composed by the He-Ne laser light of 10 miliwatts and 632 nm, a charge-coupled device (CCD) camera, and the host computer with image acquisition software. An aliquot of 10 µl of the semen sample was placed in a microscope slide and covered with a slip and then illuminated by the laser. The acquired images (512 x 512 pixels, sampling rate 0.08 seconds, shutter speed 1/60 seconds) were digitized to 8 bits and stored in the memory of the computer. For the computer analysis, three semen samples of each donor bull were evaluated using the HTM-IVOS-Ultimate, version 12.3, placing 20µl aliquots in the reading chamber.

Results and Discussion

The following variables were analyzed: average path velocity (VAP, µm / s), and straight-line velocity (VSL, µm / s). The VAP results by CASA in each bull were: A =86.6 (b), B= 91.03 (ab), C= 89.63 (ab), D= 83.43 (b), E=99.37 (a), F= 93.03 (ab). The averages followed by the same letter did not differ in the Tukey test ($P<0.05$). For VSL data were: A=69.33, B= 72.87, C= 69.47, D= 65.67, E= 67.57, F= 74.70, and IM results obtained by BSL were: A 108.6 (18.087), B 158.3 (40.549), C 168.7 (46.015), D 204.3 (68.482), E 148.8 (25.253), F 219.8 (99.294), both showing no statistical difference among AI-bulls. Despite no observed differences, bull A showed the worse IM, and low values of VAP and VSL. BSL estimates were coherent to CASA when evaluating frozen bovine semen.

References

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Morphological alterations on spermatogenesis of mice by caused BPP-11e and BPP-11eAP, two bradykinin potentiating peptides from *bothrops jararaca* venom

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Introduction

The low molecular weight fraction (LMWF) of the snake venom comprises a series of bioactive peptides, such as Bradykinin Potentiating Peptides (BPPs). One of its mechanisms of action is related to inhibition of the somatic Angiotensin Converting Enzyme (sACE). BPPs 11e (<EARPPHPPIPP) and 11e-AP (<EARPPHPPIPPAP) from *B. jararaca* venom inhibit sACE, oligopeptidases 24.15 and 24.16, with K_i at the μM level. Recently, we have shown that LMWF from *Bothrops jararaca* causes inhibition in the process of spermiogenesis, transformation of spermatid into spermatozoon, in mice spermatogenesis. These data indicated that such effects can occur via the interaction of BPPs with the testicular ACE (tACE), an enzyme expressed in germ cells exclusively during spermatogenesis (1). Studies have shown the involvement of the tACE in male fertility, in fertilization, as well as in spermatogenesis.

Materials and methods

Male Swiss mice (35g) were treated with BPP-11e or BPP11-eAP (5μg) in the left testis and vehicle in the right testis, and after 7 days the animals were killed. Testes were removed and analyzed by Mallory trichromic staining. Morphological and morphometric parameters were analyzed in approximately 50 sections using Zeiss Axioskop 2 photomicroscope. All data were presented as the mean ± SEM and the criteria for statistical significance was set at $P<0.05$ (GraphPad Prism 4.0, GraphPad Software, Incorporation).

Results and Discussion

Seminiferous tubule morphology observed in animals treated with BPP-11eAP showed an intense induced disruption of the epithelium, presence of atypical multinucleated cells in the lumen, degenerated germ cell in the adluminal compartment, when compared with morphology of the right testis. Morphometric parameters showed significant reduction ($P<0.001$) of round spermatids (7.75±1.50), spermatocytes (zygotene, 7.25±1.50; pachytene, 5.75±0.86) when compared to the right testis (29.5 ± 0.5, 15.2 ± 2.5 and 24.5±3.1, respectively). Interestingly, no morphological or morphometric alterations were observed in animals treated with BPP-11e. These data suggest that the effects can not be attributed to the interaction of BPPs with the tACE or oligopeptidases (24.15 or 24.16) because the alterations in the spermatogenesis were observed only in animals treated with BPP-11eAP. Thus, these results open perspectives for the understanding of the effects BPP-11eAP causes on spermatogenesis.

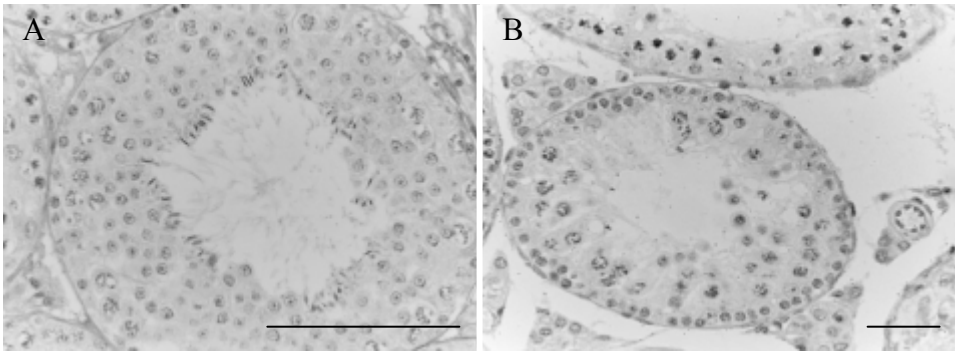

Figure 1. Effect of BBP-11e and BBP-11e AP in the spermatogenesis of male adult mice. Morphological analysis in the seminiferous epithelium of male adult mice treated with BPP-11e (A) and BPP-11eAP (B). Bars 100μm.

References:

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