Uranium effect on pregnant female rats

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Introduction

Uranium may be fed to animal as a contaminant of bi-calcium phosphate, most of its toxic effect was described at the kidney, but little is known about its effect on embryogenesis. Considering the possible ways of radionuclide contamination in the human (or animal) body, ingestion is of concerns because it is a chronic source. Uranium radionuclide is able to enter in the living cells by either metabolic or other processes giving rise to localized doses which can be high (1).

Material and Methods

In order to evaluate the uranium effect on reproduction, eight female rats (3 mo) were fed 100 ppm of uranium from after copulation until weaning. Two females were kept with a male in each cage and spermatozoa were searched in vaginal smears collected every morning. After copulation the female was kept in a separated cage until parturition and fed either a commercial rat chow doped with 100 ppm of uranyl nitrate (treated, n = 4) or a not doped (control, n = 3). Water and feed intake were evaluated daily, and the total amount taken in divided by the number of fetus/pups. Uranium feeding finished after parturition. One female of each group was euthanized and evaluated for teratogenic effect (after diaphanization) on days 4, 7, 15 and 25. Data were compared by Mann-Whitney test due to lack of normal distribution.

Results and Discussion

Uranium ingestion had no effect ($p = 0.56$) on feed consumption during pregnancy (control 1.89 ± 0.8 g/fetus; treated 1.95 ± 0.7 g/fetus); an overall toxic uranium effect was not evidenced on food ingestion at this time. There was no difference ($p = 0.46$) on daily water consumption between treated 3.78 ± 0.12 ml/fetus and control 3.67 ± 0.12 ml/fetus, if there was a uranium kidney toxic effect, as previous described (2), a water imbalance in treated animals would be expected, but that did not occur. Treated female rats had a similar number of pups (12.75 ± 0.85; $p = 0.59$) than controls (12.0 ± 1). After parturition treated female and its litter ingested less food ($p = 0.007$; 4.14 ± 0.23 g/brood) than controls (5.3 ± 0.36 g/brood). The same happened with water intake, treated rats ingested 6.69 ± 0.37 ml/brood and control ingested 8.42 ± 0.54 ml/brood ($p = 0.008$). Although there was no visible teratogenic effect either macroscopic or after diaphanization, somehow previous exposure to uranium interfered on litter metabolism from birth to 22 days of age. These results from rat studies may provide information about the effect of uranium contamination on humans. Additionally, the conditions of our experiment simulate a lifelong scenario, considering a prolonged intake of uranium-contaminated food.

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Fixed-time artificial insemination (FTAI) in *Bubalus bubalis* by employing a modified Ovsynch protocol

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Introduction

The buffalo herd in Brazil has an average annual growth of 12.5% (1). It was found that after the second treatment with GnRH, ovulation occurred between 20 and 32 hours (2). Different periods were studied and it was concluded that the best conception rates were achieved when AI was performed 16 hours after the second injection of GnRH in the Ovsynch protocol (3). The objective of this study was to determine the ideal time of using FTAI in buffalos using the Ovsynch protocol.

Material and Methods

The Ovsynch protocol (D0=GnRH, IV; D7=PGF2α, IM and D9=GnRH, IV; LH or Hcg; IV) was used to synchronize the oestrous cycle in 106 buffalos during the breeding season from April to August in 2008 and 2009 in Morretes-PR. On the 9th day of the protocol the animals were divided randomly into three groups and received GnRH (25 μg IV, n = 38); hCG (2500 IU IV, n = 35,) or LH (12.5 mg IV, n = 33). Sixteen (16) hours after the last treatment, the preovulatory follicle and corpus luteum diameters were measured using ultrasound exams and FTAI was subsequently conducted. The measurement of the corpus luteum was performed along with pregnancy diagnosis 42 days after FTAI. In 63 days the ultrasound test was done again to verify the occurrence of embryonic death.

Results and Discussion

The conception rates for the groups GnRH, hCG and LH were 39.4, 51.4 and 45.5% respectively. These findings are similar to a study that compared the conception rate of animals that received GnRH or hCG, resulting in a conception rate of 46.8% and 50.8% respectively (4). Ultrasonographic exams on the day of FTAI revealed that 90.4% of the animals had preovulatory follicles (>9 mm) and these follicles had an average of 11.9 mm in diameter. There was no significant association between groups with respect to the diameter of the corpus luteum. When comparing the association between the oestrus synchronization protocol and the probability of conception within each level of ECC (>3 or ≤3), there were no significant statistical differences. The embryonic death rate on the 63rd day after insemination was 6.25%, similar to another study that showed a rate of 7% (5).

Table 1. Mean diameter of preovulatory follicles on the day of insemination and the corpus luteum in pregnant buffalos in 42 days.

Group	Number of animals (n)	Mean diameter of follicle (mm)	Mean diameter of corpus luteum (mm)
GnRH	14/38	11.3[a]	21.9[a]
hCG	18/35	12.2[a]	21.7[a]
LH	15/33	12.5[a]	21.5[a]

[a]different letters in the same column represent statistical differences between groups.

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The effect of Leukemia Inhibitory Factor on the activation of goat preantral follicles *in vitro*

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Introduction

The requirements for the continued growth of larger preantral follicles have been extensively explored in rodents and to a less extent in domestic species. Studies using culture of isolated preantral follicles *in vitro* are important to provide a better understanding of the earliest stages of follicular development in ruminants, particularly in follicular activation (1). Leukemia inhibitory factor (LIF) was first identified as a pleiotropic cytokine of the interleukin-6 family and its identification and mRNA expression are consistent with the concept that might be involved in growth initiation of primordial follicles in primates (2-3). LIF can also enhance the survival, migration, proliferation and meiosis resumption of primordial primitive cells in rodents (4). This study was conducted to evaluate the effect of LIF on the activation and development of preantral follicles using goat ovaries cultured *in vitro*.

Material and Methods

Ovaries (n = 10) of adult goats were collected from an abattoir, washed in 70% alcohol two times and kept in a Minimum Essential Medium (MEM) with 100µg/ml penicillin and 100 µg/ml streptomycin for tissue preparation (5). Ovarian cortex was sliced in 9 mm^3 pieces and *in vitro* culture was performed at 39°C, 5% CO_2 in 1ml of α-MEM with ITS, pyruvate, glutamine, hypoxantine, BSA and LIF at 1, 10, 50, 100 or 200 ng/ml for 7 days. All samples were evaluated histologically with hematoxylin and eosin stain in 400x magnification after 7 days of culture.

Results and Discussion

The percentage of viable primordial and primary follicles did not differ when cultured in different concentrations of LIF. However, the number of primordial follicle was lower ($P < 0.05$; Figure 1A) and primary follicle was higher ($P < 0.05$; Figure 1A) when the follicles were cultured in 10 or 50 ng/mL for 7 days than in the positive (non cultured) and negative (MEM alone) control. In rodents, LIF maintained the viability and allowed cryopreservation of the preantral follicles (6). Our findings suggest that LIF induces follicular activation and the preantral follicles advanced to subsequent stages after 7 days of culture. Representative histological aspects of viable primordial and primary follicles are showed in Figure 1C. In conclusion, we suggest that LIF plays an important role on the activation and survival of goat preantral follicles cultured *in vitro* for 7 days.

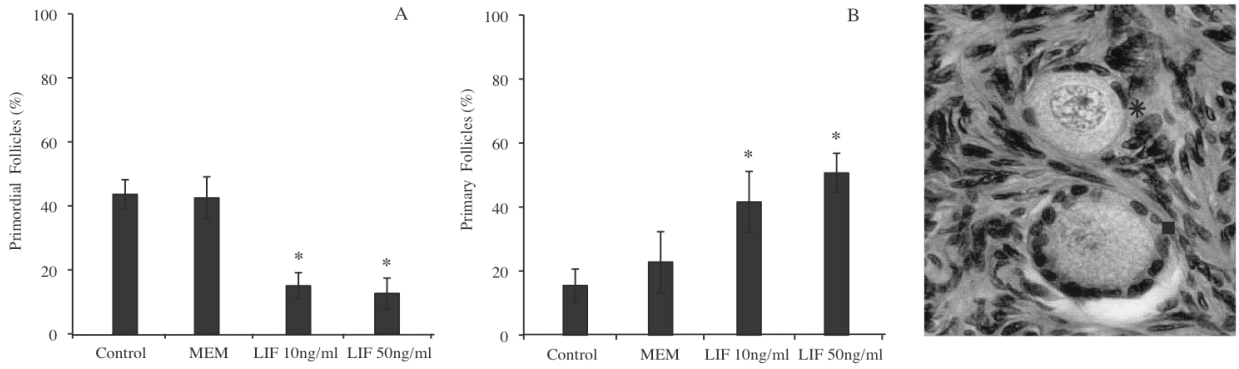

Figure 1: Percentage of primordial (A) and primary (B) follicles cultured *in vitro* with LIF. (*) Difference between LIF treated groups and control and MEM groups ($P < 0.05$). (C) Primordial [✳] and primary follicle [■].

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The NADPH oxidase complex in human uterine leiomyomas

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Introduction

Uterine leiomyomas or fibroids are benign tumors that affect over 70% of women and are characterized by increased proliferation of smooth muscle cells (SMCs) as well as increased deposition of collagens type I and III. Our previous work indicates that NADPH oxidase-derived reactive oxygen species (ROS) play an important role in the platelet-derived growth factor (PDGF) proliferative pathway in leiomyoma SMCs. Specifically, ROS can act as signaling molecules by modifying effectors such as protein tyrosine phosphatases (PTPases). The goals of our study were to 1) determine the expression pattern of the components of the ROS-generating enzymatic complex, NADPH oxidase, in leiomyoma and myometrial SMCs; 2) study the mechanism of activation of the complex in these two cell types; and 3) determine whether ROS can affect PTPase activity and whether changes in PTPase activity might regulate proliferation of myometrial and leiomyoma SMCs.

Materials and Methods

Primary cultures of normal myometrial and leiomyoma SMCs were used as our experimental model. We performed qRT-PCR to assess mRNA levels and immunohistochemistry (IHC) to assess protein localization in tissue samples. The mechanism of activation was studied by focusing on phosphorylation of the cytosolic NADPH oxidase subunit, $p47^{phox}$, using both immunofluorescence staining and immunoblotting following treatment with PDGF. Manipulation of PKC activity by the PKC inducer PMA and PKC inhibitors Bis and Go-6983 was used to determine the involvement of PKC in PDGF-induced ROS production and Erk activation. PTPase activity in cell lysates of treated cells was measured using a colorimetric tyrosine phosphatase assay. The PTPase inhibitor sodium orthovanadate (VAN) was used to assess the involvement of these enzymes in cell proliferation. Cell proliferation was measured by tritiated thymidine incorporation assays.

Results and Discussion

Our results showed that several genes encoding NADPH oxidase complex components are expressed in leiomyoma and myometrial tissues, and that $p47^{phox}$ protein is also present in both tissue types. Overall gene expression showed downregulation of $p47^{phox}$ and catalase in leiomyomas. Moreover, IHC suggested a higher nuclear expression of $p47^{phox}$ in leiomyoma SMCs. The importance of lipid rafts in NADPH complex activation was supported by results showing that PDGF treatment induced $p47^{phox}$ enrichment within lipid rafts. Moreover, stimulation of PKC with PMA led to ROS production and Erk phosphorylation, whereas inhibition of PKC reduced PDGF-induced ROS production and Erk phosphorylation. In regards to potential targets of ROS action, treatment with an exogenous source of ROS, hydrogen peroxide (H_2O_2), was sufficient to inhibit PTPase activity in leiomyoma SMCs, but not in myometrial cells. No effect of PDGF on PTPase activity was observed in either cell type. Inhibition of PTPase activity by low concentrations of VAN led to an induction of cell proliferation. Interestingly, leiomyoma SMCs were more sensitive to VAN than myometrial SMCs. Our results show that 1) activation of the NADPH oxidase complex by PDGF involves activation of PKC; 2) PTPase activity is important for SMC proliferation; and 3) PTPases are sensitive to regulation by H_2O_2 in leiomyoma SMCs, but not in myometrial SMCs. In addition, PTPases are more sensitive to inhibition by VAN in leiomyoma SMCs than myometrial SMCs. The lower catalase gene expression in leiomyoma SMCs suggests a decreased ability of leiomyoma SMCs to scavenge ROS rendering this cell type more sensitive to a buildup of ROS levels and leading to a compensatory decrease in $p47^{phox}$.

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Effect of fluxinin meglumin, recombinant bovine somatotropin and/or human chorionic gonadotropin in reducing embryo mortality in nelore cows (*Bos taurus indicus*)

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Introduction

Pharmacologic strategies have been employed to reduce the embryonic mortality in cattle (2, 4). Aim was to compare the effect of Flunixin Meglumine (FM), Recombinant Bovine Somatotropin (bST) and/or Human Corionic Gonadotropin (hCG) on the reduction of embryonic mortality in Nelore cows between the 15 and 19 days of gestation. The hipothesis is that cows treated with FM, bST and/or hCG show lower embryo mortality and greater conception rates.

Material and Methods

Lactating Nelore cows (n = 975), 35 to 70 days postpartum, received on D-10 an intravaginal device containing 1g of Progesterone (DIB®) associated with a injection of 2mg of estradiol benzoate (Gonadiol®), i.m. On D -2 the device was removed and animals received one injection of 112,5µg of D-Cloprostenol (Preloban®); 300UI of Equine Chorionic Gonadotropin (Folligon®) and 1mg of Estradiol Cipionate (ECP®) i.m. After 48 hours from the last injection (D0), cows received Fixed Timed Artificial Insemination (FTAI) and seven days later were divided equitably in eight groups to receive one of the following treatments: nothing (Group Control; n = 124); 2,2mg/Kg of FM (Banamine®), i.m. on D16 (Group FM; n=122); 500mg of bST (Boostin®) s.c. on D7 (Group bST; n = 119); bST on D7 + FM on D16 (Group bST/FM; n = 121); 2.500UI of hCG (Chorulon®) i.m. on D7 (Group hCG; n = 124); hCG on D7 + FM on D16 (Group hCG/FM; n = 124); bST and hCG on D7 (Group bST/hCG; n = 120) or bST and hCG on D7 + FM on D16 (Group bST/hCG/FM; n = 121). Blood samples were collected on D7 and D16, to measure plasmatic progesterone (P_4) concentration through radioimunnoassay. Pregnancy diagnosis was performed 40 days after FTAI by ultrasound. Pregnancy rates were analyzed by logistic regression using the GLIMMIX procedure of SAS and the values of P_4 were transformed to square root and analyzed by ANOVA (PROC GLM).

Results and Discussion

There was no difference in conception rates for different treatments (p = 0.4995), which were 57.26%, 47.54%, 60.50%, 56.20%, 59.68%, 64.52 %, 63.33% and 64.46% for groups Control, FM, bST, bST/FM, hCG, hCG/FM, bST and bST/hCG/FM, respectively. However, when the conception rate was evaluated considering all the females treated or not with hCG, there was a higher rate of conception on treated vs. non-treated cows (62.99% *vs*. 55.35% respectively, p = 0.01). This difference was not observed among females treated or untreated with bST (61.12% *vs.* 57.29% respectively, p = 0.21) nor FM (58.20% *vs.* 60, 16% respectively, p = 0.46). Plasma concentrations of P_4 on D7 and on D16 did not differ (p = 0.9081 and p = 0.0888, respectively) among the different groups. Concentrations of P_4 on D7 did not differ (p > 0.05) among non-pregnant cows and heifers treated with hCG, bST or FM. On D16, for females treated with hCG, the concentrations of P_4 were greater for pregnant compared to non-pregnant females (10.62 ± 0.39 *vs*. 9.55 ± 0.37, respectively; p = 0.0503). It was concluded that cows treated with hCG 2.500UI seven days after TAI have lower embryo mortality and higher conception rates at 40 days of gestation.

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Somatic cells proliferation in the sexually mature bullfrog testis (*Lithobates catesbeianus*)

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Introduction

Spermatogenesis is one of the most efficient self-renewing process, resulting in the daily production of million of spermatozoa per unit (gram) of testis parenchyma. Although there are some particularities, this process seems to be fairly conserved among vertebrates and the physical and biochemical interactions of somatic and germ cells are crucial in creating an ideal microenvironment for the proper germ cells development (1). The bullfrog (*Lithobates catesbeianus*) is a North American anuran introduced in Brazil several decades ago due to its substantial economic importance. This species has also been used as an experimental model for biological research, including important aspects related to reproductive biology (2). However, the knowledge regarding the testis structure and function in this amphibian species is still scarce. In this regard, the main objectives of the present study were to investigate the proliferative activity of the testicular somatic elements [Sertoli cells (SC); Leydig cells (LC); and peritubular myoid cells (PMC)], and to correlate this variable with germ cells development.

Materials and Methods

Nine sexually mature bullfrogs received a single intracoelomic injection of tritiated thymidine (1µCi/g/BW). Two hours after injection the animals were anesthetized and had their testes removed, longitudinally and transversally cut, in order to obtain six representative fragments from the entire testis. These fragments were fixed by immersion in 4% buffered glutaraldehyde, routinely prepared and embedded in glycol methacrylate to perform autoradiographic and histomorphometric evaluations.

Results and Discussion

Considering the six different regions evaluated, no significant differences were found for the somatic elements mitotic index. These important results suggested that the testis parenchyma in the bullfrog presents an isometric distribution. In relation to the spermatogenic cysts, SC proliferation was found preferentially associated with spermatogonia, particularly type B. This result strongly suggests that SC must proliferate in order to ensure an adequate number of this key somatic cell that, according to the literature (3), is crucial to support the dramatic cyst growth that occurs during this phase of spermatogenesis. The LC and PMC proliferation index was usually higher ($p > 0.05$), when these cells were nearby or in the proximity of late spermiogenenic cysts. At the present moment we do not have a clear explanation for these findings. However it is already known that these cells play an important role during spermiation, particularly androgens secreted by LC (4). In order to better comprehend the testis structure and spermatogenesis and the interaction between the somatic and germ cells elements in the bullfrog we are currently performing ultrastructural and light microscopy studies.

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Masculinization of female gerbil by exposure to testosterone

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Introduction

Changes induced by steroid hormones during development are often irreversible and may affect organs of the reproductive, nervous and immune systems (1). It is well know that development is an event coordinated by epigenetic events which are mediated by a cascade of cell signaling (1). Some compounds like the endocrine-disrupting chemicals (EDCs) have the capability of mimicking some steroids hormones and alter these cascades of cell signaling affecting the pattern of gene expression during the critical periods of organogenesis. Researches have demonstrated that prostatic diseases have origin in early life, being the abnormal androgens exposure the major risk factor to the appearance of late diseases, like benign prostatic hyperplasia (BPH) and prostatic cancer (Pca; 2). In this manner, the aim of this work was to study the effects of androgenic exposure during embryonic phase on the prostate of gerbil adult female.

Material and Methods

Pregnant adult female gerbils (*Meriones unguiculatus*) were exposed to testosterone cypionate (100 µg, TGB-group; 1 mg, TGA-group), during gestation (days 17-18) by subcutaneous injection. The female offspring from these pregnant females were maintained in the polyethylene boxes, receiving water and food normally, until 4 months of age. The prostatic glands of adult animals (Control - C, TGB and TGA) were processed for light microscopy. Histological sections were stained by haematoxylin-eosin for morphological studies. Anogenital distance (AGD) and stereological data were obtained from these groups (values are means ± standard error; statistical analysis – ANOVA, Tukey, $p \leq 0.05$). Immunohistochemistry was performed for ER-α.

Results and Discussion

Females from TGA group were masculinized showing increased AGD and prostatic acini either around the bladder neck or around the vaginal wall, characteristics not observed in the C and TGB groups. Values of AGD were higher in TGA group (3.7 mm ± 0.4) in comparison with C (1.7 mm ± 0.1) and TGB (2.2 mm ± 0.2) groups. Stereological data showed a significant increase of stromal area at TGA group (50% ± 2) in comparison with C (37% ± 1.7) and TGB (30% ± 2.1) groups. According to (3) prostatic stromal increase is common in BPH due to epithelial-mesenchymal transition (EMT). Moreover, a significant decrease of luminal area was observed at prostate of TGA (21% ± 1.7) group in comparison with C (37% ± 2.2) and TGB (38% ± 2.4) groups. Significant differences were not observed for stereological epithelial data between the groups. The prostatic glands of TGA group showed an expressive reaction for ER-α both on the stromal and epithelial compartments, fact which agree with some researches (4, 5) is associated with ER-α pathway in the development of prostatic diseases. These results showed some of the effects that the abnormal testosterone exposure during prostatic embryogenesis may have on prostate health in gerbil adult life, being a useful method for studying the potential that EDCs, with androgenic action, may have on this gland.

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Angiotensin II profile and mRNA encoding RAS (Renin-Angiotensin System) proteins during bovine follicular wave

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Introduction

According to a new concept of local renin-angiotensin system (RAS), all components of RAS are produced and regulated in the Angiotensin II (AngII) target issue. Moreover, it was demonstrated that AngII can be secreted into follicular fluid even in perfused ovaries in vitro, suggesting presence of an ovarian RAS. The objective of the present study was to characterize the AngII profile and mRNA encoding RAS proteins during antral follicular development.

Materials and Methods

Cows were ovariectomized on days 2, 3 or 4 of the follicular wave (4 cows per day). Therefore, with this experimental design, samples were collected before deviation (day 2) and with difference (day 3) or marked difference (day 4) on follicular size between first and second largest follicles. Follicular fluid and cells from these two largest follicles were recovered to determine AngII concentration and mRNA abundance of genes encoding angiotensin converting enzyme (ACE), (pro) renin receptor and renin binding protein (RnBP).

Results and Discussion

The concentration of AngII in follicular fluid increased in dominant follicle at time expected for follicular deviation (Fig. 1). However, no regulation was observed in second largest follicle (data not shown). The profile of RAS-related genes is shown in Fig. 2. In conclusion, the rise in the concentration of AngII in dominant follicle during and after follicular deviation supports our recent finding that AngII has a role on FSH-independent stages of follicular growth. Moreover, for the first time, the expression and regulation of (pro)renin receptor and RnBP was demonstrated during antral follicular development, suggesting a role of these enzymes regulating ovarian RAS.

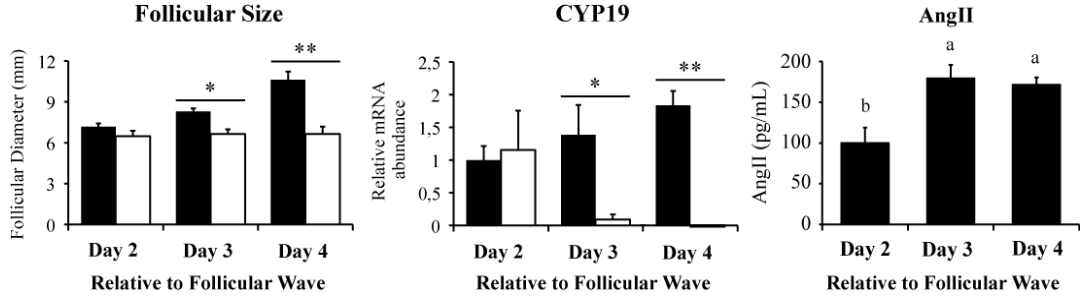

Figure 1. Follicular diameter and aromatase (CYP19) relative mRNA abundance in largest (black bar) and second largest (open bar) follicle (mean ± s.e.m.) and AngII concentration in follicular fluid during development of the largest follicle. Asterisk (* or **) indicates statistical difference between largest and second largest follicle accessed by paired Student's T test using cow as subject. * $p < 0.05$; ** $p < 0.001$. a≠b $p < 0.05$.

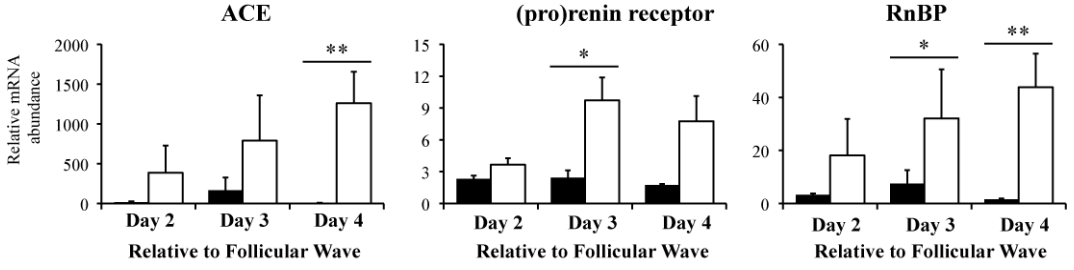

Figure 2. Expression of renin-angiotensin system related genes in granulosa cells recovered from largest (black bar) and second largest (open bar) follicle (mean ± s.e.m). Asterisk (* or **) indicates statistical difference between largest and second largest follicle. * $p < 0.05$; ** $p < 0.001$.

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High-fat diet impairs the sperm production in rat

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Introduction

Obesity disrupts sex hormone secretion and impairs semen quality and fertility (1). In order to improve the knowledge about the mechanisms responsible for these alterations, the present study evaluated the effect of high fat diet-induced obesity on sperm production and epididymal transit in rats.

Material and Methods

Adult male Wistar rats were fed balanced (*Control*, 4% fat) or high-fat diet (*Obese*, 20% fat). After 17 weeks, rats were killed and testis and epididymis were removed. The following parameters were evaluated: plasma testosterone (T) and insulin concentration, insulin tolerance test (ipITT), daily sperm production (DSP) per testis, sperm number and sperm transit time in the epididymis. The distribution of androgen receptor (AR) in the testis was analyzed by immunohistochemistry and its protein content by Western blotting.

Results and Discussion

Biometric and metabolic alterations observed in obese rats are shown in Table 1. These rats showed an increase in the epididymal and retroperitoneal fat deposits, and hyperinsulinemia. Moreover, ipITT analysis demonstrated that even after the insulin administration, blood glucose levels remained high in obese animals, confirming the state of insulin resistance. Obesity did not affect testicular and epididymal weight and gonad-somatic index (IGS). However, the DSP, and sperm counts in the caput/corpus and cauda epididymis were significantly decreased after treatment with high-fat diet. On the other hand, sperm transit time in the epididymis was not altered. The plasma T concentration was reduced by about 30% in obese animals. Immunocytochemistry and Western blotting analysis showed no variation in AR in the testis (Fig. 1). The alterations in DSP and epididymis sperm number caused by obesity were more severe in comparison to other situations of T reduction and inefficient insulin action such as the experimental diabetes (2). Our data corroborate previous studies which report that obesity is associated with reduced male fertility. Thus, it is possible that the negative effects abovementioned were also influenced by other paracrine factors which are altered in obese animals. These factors will be investigated later.

Table 1. Metabolic and biometric parameters and sperm counts of control (C) and obese (O) rats.

Parameters	C	O
Body weight (g)	467 ± 14.4	556 ± 20.3*
Epididymal fat deposits (mg)	7.6 ± 1.7	14.8 ± 1.4*
Retroperitoneal fat deposits (mg)	6.9 ± 1.6	16.2 ± 1.7*
Insulin (ng/ml)	3.4 ± 1.2	5.1 ± 0.8*
Testis Absolute weight (g)	1.8 ± 0.2	1.8 ± 0.1
Epididymis Absolute weight (g)	0.7 ± 0.1	0.8 ± 0.1
IGS	0.73 ± 0.04	0.66 ± 0.03
Testosterone (ng/dl)	340 ± 59	103 ± 21*
DSP (x10^6/testis/day)	28.2 ± 0.7	19.3 ± 2.9*
Caput/corpus epididymis sperm number (x10^6/organ)	98.8 ± 1.6	64 ± 6.1*
Cauda epididymis sperm number (x10^6/organ)	289 ± 25	181 ± 18*
Epididymal sperm transit time (days)		
Caput/corpus	3.5 ± 0.1	3.6 ± 0.8
Cauda	10.2 ± 0.6	9.7 ± 1.5

Values represent Mean ± S.E.M. * Significant difference compared to control ($P \leq 0.05$).

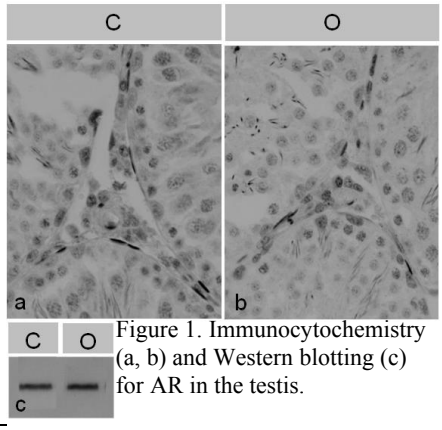

Figure 1. Immunocytochemistry (a, b) and Western blotting (c) for AR in the testis.

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Sterilization of dogs with intratesticular injection of a zinc-based solution - Histophatological evaluation of acute and chronic phase

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Introduction

Spermatogenesis is an elaborate process of germ cell proliferation and differentiation that leads to the production and release of spermatozoa from the testis (1). A zinc-based solution, pH neutralized in BioRelease Technologies physiological vehicle, was developed (B.E.T. Labs, Lexington, Kentucky, USA) as a chemical sterilant for intratesticular injection in male dogs. The aim of this study was to evaluate, by light microscopy analysis, the acute and chronic phase of a single intratesticular injection of zinc-based solution in male dogs.

Materials and Methods

Twenty mongrel dogs, ranging from puberty to 5 year old, were assigned to 3 treatment groups. Group 1 (n = 10), the control placebo, was injected with saline solution and orchiectomy happened 15 days (n = 5) and 150 days (n = 5) post injection. Group 2 (n = 5), were injected with zinc-based solution and all dogs were castrated 15 days post injection and Group 3 (n = 5), were injected with zinc-based solution and all dogs were castrated 150 days post injection. The dosage of zinc-based or saline solution was based on testicular width, determined by a caliper. Testis and epididymis were removed, fixed in glutaraldehyde, embedded in methacrylate, sectioned and stained with toluidine blue for histophatological examination (2).

Results and Discussion

Histophatological evaluation of acute phase of testis of treated dogs revealed germ cell degeneration, vacuolation of Sertoli cell, fewer germ cells and formation of multinucleated giant cells in atrophic seminiferous tubules. Areas close to the site of injection had severe scar tissue formation with fibroblasts and collagen in the intertubular region. In addition, there was interstitial hemorrhage and increased number of inflammatory cells. There were no morphological changes distal to the site of injection. In control group the changes were comparable to the treated group, although there was no evidence of inflammatory reaction or scar tissue formation. Histopathological evaluation of chronic phase revealed that there were no changes detected in testes from control dogs. Examination of treated group revealed germ cell degeneration, Sertoli cell vacuolation, fewer germ cells, formation of multinucleated giant cells and a lack of elongated spermatids in atrophic seminiferous tubules. Leydig cells had varying degrees of steatosis. The majority of seminiferous tubules in all zinc-treated dogs were lined only by Sertoli cells, which were vacuolated, with a complete absence of spermatogonia. Although there were less severe morphological changes distal to the site of injection, spermatogenesis appeared to be substantially impaired (i.e. smaller germ cell numbers with rare scattered round spermatids, which were apparently not viable). Histological findings in the present study demonstrated that intratesticular injection of the zinc-based solution Testoblock impaired spermatogenesis. This suggests that the zinc-based solution Testoblock is effective as a chemical sterilant for dogs (2). However, further trial has been developed to establish the long-term irreversibility of effects on the seminiferous epithelium.

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Gene expression of canine prostate specific esterase (CPSE), prostatic acid phosphatase (PAP) and prostate specific antigen (PSA) in dogs

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Introduction

The prostate is the only accessory sex gland in the canine genital system and it can spontaneously develop benign prostatic hyperplasia and prostatic adenocarcinoma, similar to what is observed in humans (1, 2, 3, 4). There are controversies about the use of prostate glycoproteins for diagnosis of canine prostate diseases. Due to the absence of a tissue marker to assess the canine prostate, this study was conducted to evaluate the expression of prostatic acid phosphatase (PAP), prostate specific antigen (PSA) and canine prostate specific esterase (CPSE) expression in healthy male dogs at different ages and dogs with prostate alterations detected by ultrasonography.

Material and Methods

Forty-seven intact male dogs were divided into groups, according to the age: young adult dogs (A group), middle aged dogs (B group) and old dogs (C group). Ten animals with prostatic alterations, as evaluated by ultrasonography were grouped separately (D group). Before the necropsy, the animals were examined using transrectal ultrasonography and, after that, the prostate was removed and samples were collected for histopathology, immunohistochemistry and RT-PCR analysis. Primary antibodies were used to bind to PSA (monoclonal mouse anti-PSA) and PAP (polyclonal rabbit anti-PAP). After the incubation with the secondary antibody and streptavidin-biotin-peroxidase solution, the samples were analyzed by optic microscopy. The RT-PCR was performed to evaluate CPSE, PAP and PSA expression, using primers designed by Primer 3 program. The gene sequences were obtained from Genbank (Y00751 CPSE, XM 542782 PAP e XM 533980 PSA). The results from PCR amplifications were analyzed using a software to measure the intensity of the bands detected (Image Aide 3.06.04 Analysis Software – Spectronics Corporation), that represent PCR product quantities.

Results and Discussion

The histopathological exam revealed that all samples from groups B, C and D had prostatic lesion in which benign prostatic hyperplasia associated with non-bacterial prostatitis was detected more often. Anti-PSA immunoreactions were weak in the prostatic tissue from animals of groups A and B and absent in samples from groups C and D. All samples presented positive staining to PAP in the apical cell surface of the prostatic epithelium. The RT-PCR showed positive amplification of CPSE and PAP, but not PSA in all samples studied. The absence of detectable PCR products from canine PSA agrees with other research (5, 6), which suggested that PSA is not expressed in the canine prostate. No difference was found between the amplicon quantities of CPSE between groups A, B and C. However, this quantity was higher in group D, when compared to the other groups. There was no statistical difference in PAP amplicon quantities among the groups. No correlation was found between CPSE and PAP amplicon levels, or between these quantities and prostatic dimensions obtained by ultrasonography, suggesting that regulation of these genes occur by different mechanisms. Moreover, there must be other mechanisms that interfere on the expression of these genes beside prostatemegaly.

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Mast cells and ethanol: interactions in the prostate, epididymis and testis of UChB rats (10% ethanol voluntary intake)

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Introduction

Alcoholism has reached alarming proportions throughout the world, but there is little research on the ethanol inflammatory effect in the reproductive organs. The mast cell, which is one of the main cells involved in inflammatory processes, is known to have two subtypes in rodents, connective (CTMC) and mucosal (MMC) tissue (1). UChB rats is a lineage of ethanol-preferring rats selected at the University of Chile from inbreeding of Wistar rats (2), representing a special model for understanding the basis of alcoholism-linked characteristics such as those found at alcohol-related human diseases. Hence, the aim of this work is to locate and quantify mast cells in the prostate ventral lobe, in the testis and epididymis of UChB rats and then assess if there is a relation between ethanol ingestion and the mast cells number in these organs.

Materials and Methods

Rats were divided into three experimental groups (n = 10/group): UChB rats that received 10% ethanol administration (UChBEtOH), UChB rats without ethanol administration (UChB) and Wistar rats (C). Samples of the prostate ventral lobe, testis and epididymis were collected after 180 days of experiments and processed for light microscopy analysis. The material was stained with toluidine blue for identification of total mast cells and immunohistochemistry was performed for CTMC identification.

Results and Discussion

The rats which received ethanol exhibited total mast cells high density (intact and degranulated) in the ventral prostate, similarly to human prostate cancer, where it may be protecting against tumor (3). There was not statistic difference in the number of mast cells, intact and degranulated, in the testis. The epididymis showed differences according to the analyzed segment. There was a higher density of intact mast cells at the initial segment and degranulated mast cells in the initial segment and cauda in UChBEtOH compared to UChB. This was, in contrast with animals receiving steroids prenatally, in which it was not verified an increase in this cell (4). Also, a higher number of intact mast cells in epididymis initial segment and caput in C compared to UChB was observed. Regarding to immunohistochemistry, there was a CTMC high density at epididymis in UChBEtOH and C. The MMC higher estimative was found in prostate and epididymis, indicating a probable inflammatory response in these organs (5).

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Diet influence on male reproductive system of Morada Nova rams

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Introduction

In Brazil, sheep that have no wool are the main genotypes due to their exceptional adaptability to the Tropics. However, little is known about the relationships between nutrition and reproduction on these animals, and this information is crucial for improving the animal production systems. Moreover, studies suggest that testis size and sperm production may be affected by modifications on protein intake from diet. Considering the importance of Morada Nova rams in the Brazilian Northeast, our aim was to study the effect of different sources of dietary protein on reproductive parameters, seminal plasma proteins and semen criteria on these rams.

Material and Methods

Twenty Morada Nova rams (average age: 184.6 ± 4.6 days and body weigh: 14.6 ± 0.6 kg) were divided into 4 groups according to the source of dietary protein: (1) soy; (2) urea; (3) leucaena (*Leucaena leucocephala*) hay; (4) cotton cake. Feeding diets were planned according to the NRC (2007) standards for sheep, to result in a daily weight gain of 125 g/animal. Scrotal parameters such as circumference (SC), width (WT), thickness (TT) and length (LT) were evaluated biweekly, between 184 and 294 day. Testicular volume (TV) was estimated according to the formula, $VT = 1/6\pi \times LT \times WT^2$. When the animals reached puberty, the semen of each animal was collected by electroejaculation. After collection, the ejaculates were assessed for volume, sperm concentration, total number of spermatozoa per ejaculate (volume x sperm concentration) and turbulence, individual progressive motility and sperm motility. Sperm concentration was measured using a haemocytometer. The morphological changes were classified into major and minor defects, after staining with bromophenol blue. Also, samples containing 400 μg of total protein from seminal plasma were electrophocused (13 cm IPG strips; pH 4 to 7), followed by SDS-2-D-PAGE (15%) and the analysis of Coommasie-stained gels was performed using PDQuest software. Differences in morphometric measurements and protein intensity among the four groups were evaluated by one-way ANOVA ($P < 0.05$).

Results and Discussion

The biometric and morphometric variables evaluated resulted in similar results for the different diets, suggesting that the different protein diets did not influence the animal development and scrotal (testis and epididymal cauda) parameters. Ejaculates presented similar average motile sperm, volume, concentration, turbulence and individual progressive motility. However, analysis of semen parameters revealed that sperm progressive motility was impaired when animals were fed leucaena hay and cotton cake. It is important to note that this diet presented the same isoproteic and isoenergetic conditions as the remaining two diets. Furthermore, seminal plasma proteins were investigated by 2D eletroctrophoresis gel and the proteins in the molecular mass region of 10 kDa to 105 kDa, and the pH range between 4 and 7, were included in comparative analyses. The identity of spots detected in the present study is unknown; however, they are identified considering the similarities between the protein map of Santa Ines and Morada Nova rams, as previously identified by our group (1). Three spots were identified as RSVP-14, RSVP-22, proteins secreted by accessory sex glands and ampula that have functions on sperm capacitation, and Bodhesin-2. Interestingly, RSVP-22 and Bodhesin-2 expression were enhanced when animals were fed leucaena, compared to the other groups, suggesting their participation on sperm vigor. These results are preliminary and additional analyses of the proteins involved on sperm parameters that are influenced by different proteic sources are required. In addition, the influence of diet on sperm function and proteins have not been described in the male reproductive tract, and their importance in this system remains to be established. In this way, these studies with tropically adapted rams could contribute to a better understanding of reproductive physiology, and factors involved in the sperm maturation and function, providing the basis for understanding aspects of these animals and their fertility potential.

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The effect of dexamethasone on bull fertility

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Introduction

Dexamethasone is a synthetic, glucocorticoid class of steroid drugs, widely used in the treatment of inflammatory and allergic reactions and immunomediated diseases. Besides these actions, dexamethasone promotes hyperglycaemia, sodium retention and potassium excretion, increases protein and lipid metabolism and is a potent diuretic. In the area of animal reproduction, this drug can cause abortion, placenta retention, dysfunction of the estrous cycle and a decrease in male fertility. The aim of this study was to evaluate the effect of dexamethasone on bull's fertility (1).

Material and Methods

The experiment was conducted during a period of 18 weeks and divided into two phases: preliminary and experimental. During of the preliminary phase of the experiment, ejaculates was collected from each bull (n = 6) once per week, for 9 weeks, with the objective of evaluating the seminal profile during spermatogenesis. The biochemical exams were performed weekly during the period of 5 weeks. Also, blood samples from the bulls were analyzed to determine the concentration of LH and testosterone. This evaluation was done weekly for four weeks. Subsequently, these exams were done in four consecutive days until the first day of administration of dexamethasone. In the experimental phase the animals (n = 6) were divided into two groups: an experimental and a control group, consisting of three animals per group. The experimental group received daily administration of 20 mg IM of dexamethasone for five days and the control group received an equivalent dose of 0.9% saline solution. In both groups an ejaculate was collected from each bull once a week for nine weeks to evaluate the seminal profile. Biochemical tests of the semen were done for 10 weeks. To measure the blood testosterone levels in the animals in the experimental phase, blood samples were collected during the five days corresponding to the application of dexamethasone.

Results and Discussion

During the four months of the experiment, including the period before and after treatment, no change in sexual behaviour was observed in the bulls. Also, during this time period there were no changes in testicular consistency, size and symmetry between the two testes of the same animal. Other variables such as: fructose, citric acid, semen concentration and morphology were assessed using the statistic parametric ANOVA test. No statistical difference was observed between the control and experimental groups before and after treatment. However, the present study demonstrated that there was a decrease in the blood concentration of testosterone, 8.18 ng/ml before treatment and 3.11 ng/ml after treatment. Similar results were also observed in studies performed with dogs (2) and marsupials (3). In contrast, no significant difference in blood plasma concentrations of testosterone was found in sheep (4).

Conclusions

Based on the data found in the present study it can be concluded that the use of dexamethasone resulted in decreased blood concentration of testosterone. However, for other parameters evaluated such as libido, testis morphology, seminal plasma, volume and pH of the ejaculate, vigour and morphological defects of the spermatozoids; no significant effects were observed.

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Effects of electromagnetic field on rat testicular development: histomorphometric analysis

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Introduction

Nowadays, there was an increasingly exposition of humans and animals to low frequency electromagnetic fields (EMF), mainly from electricity distribution networks and electro-electronic devices. The present study aims to evaluate the extension of possible effects of EMF on testicular development.

Materials and Methods

Pregnant Wistar rats (*Rattus norvegicus, var. Albinos*) were separated into two groups, a control (n=5) and exposed to EMF from the 13th day of gestation (n = 7). After birth, male pups were randomly chosen and continued to be exposed to EMF until 21st postnatal day (n = 12); or maintained in the same experimental conditions, except exposure to EMF (control group / n = 12). The treated group was exposed to EMF with frequency of 60 Hz and magnetic flux intensity of 1 mT, irradiated by a series of Helmholtz coils (PHYWE - GERMANY). Testis were fixated and embedded in glicolmetacrilate resin. Slices with 4µm thickness were stained by toluidine blue/sodium borate 1%. Morphometric analyses were performed according to Silva Jr et al. (1). Plasma testosterone analysis was performed according to Brown et al. (2; n = 6).

Results and Discussion

No pathological changes were observed in animals exposed to EMF. Table 1 shows that parameters related with seminiferous tubules were reduced in animals exposed to EMF. Total Leydig cell volume was diminished in animals exposed but testosterone level was not affected. Previous studies reported effects caused by electric and magnetic fields, such as adverse effect on sperm quality in human (3). In the present study, histomorphometric analysis showed that exposure to EMF can delay the testicular development, slowing the transition of non-lumined spermatic cords to lumined seminiferous tubules.

Table 1. Histomorphometric parameters and volume (µm^3) of the testicular components (mean ± SD).

	Control (n = 12)	Exposed to EMF (n = 12)	P
Tubular length (m)	5.617 ± 0.530	6.167 ± 1.008	P = 0.108
Sertoli cells per Cross section	13.792 ± 1.075	12.990 ± 0.988	P = 0.070
Sertoli cells population (x107)	1.939 ± 0,124	2.017 ± 0.227	P = 0.578
Tubular diameter (µm)	155.10 ± 3.463	141.48 ± 4.954	P < 0.001*
Seminiferous tubules (µm^3)	106.422 ± 7.645	96.816 ± 1.402	P = 0.048*
Seminiferous epithelium (µm^3)	92.835 ± 7.788	83.88 ± 12.63	P = 0.048*
Lumen (µm^3)	7.270 ± 3.025	4.110 ± 1.363	P = 0.034*
Tunica propria (µm^3)	8.007 ± 1.538	8.820 ± 2.746	P = 0.058
Intertubular space (µm^3)	10.985 ± 2.657	11.694 ± 1.957	P = 0.462
Leydig cells (µm^3)	3.011 ± 0.481	2.225 ± 0.584	P = 0.001*
Connective tissue cells (µm^3)	0.943 ± 0.301	1.263 ± 0.365	P = 0.029*
Blood vessels (µm^3)	0.714 ± 0.278	1.014 ± 0.379	P = 0.024*
Lymphatic space (µm^3)	6.094 ± 2.702	6.791 ± 1.852	P = 0.468
Testosterone levels (ng/dL)	3.93 ± 0.88	3.73 ± 1.01	P = 0.69

* Statistically significant (p < 0.05).

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Profile SDS-PAGE of seminal plasma in Brangus and Brown-Swiss bulls

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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 bull`s fertility (2). Peptides of 55 and 66 KDa were present in bulls with excellent spermatic conditions for example motility and vigor (3). On the other hand, 13 and 33 KDa peptides were observed in association with unfavourable spermatic conditions (3). The objective of this study was to determine the profile SDS-PAGE of seminal plasma and evaluate the semen characteristics in Brangus and Brown-Swiss bulls.

Material and Methods

Semen from eight Brown-Swiss and 14 Brangus, 36 months old, were collected by electroejaculation during summer of 2009-2010. From each breed (Brown-Swiss and Brangus respectively) a total of 48 and 84 semen samples were collected in an interval of 14 days. Semen volume, motility, vigor, major defects and minor defects were evaluated according to Brazilian College of Animal Reproduction (6). Animals were divided in two groups: poor semen (motility <50% and major defects >10%) and good semen, and subsequently compared regarding the composition of seminal plasma proteins. Samples of seminal plasma were centrifuged (1500g/15 min) 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 Bradford (4) and electrophoresis was performed according to Laemmli (5). Gels were fixed with isopropanol: acetic acid: water (4:1:5 v/v) for 30 minutes, and stained in the same solution containing 2% of Comassie Blue R250. Each semen collection was used in duplicate. The concentration of proteins was measured using a spectrophotometer PF-901(Chemistry Analyser Labsystems). Gels were submitted to a photodocumentation system (Bio Doc-IT and Visidoc-IT Gel Documentation systems, UVP) and analysed by Doc-IT-LS 6.0 software. GLM from SAS, version 6, was used in order to evaluate possible variations of seminal variables and protein molecular mass. Statistical significance was accepted from $P < 0.05\%$.

Results and Discussion

The means of semen variables in Brangus and Brown-Swiss were respectively: volume (5 ± 1 and 6 ± 1 mL), motility (75 ± 5 and 78 ± 4%), vigor (4 and 4), major defects (7 ± 2 and 6 ± 3%) and minor defects (12 ± 4 and 13±3%). The results of analyses of gels revealed a variety of proteins in each animal and among bulls. There were 28 different major polypeptides, ranging from 15 to 24 bands in each individual bull. In two Brown-Swiss and six Brangus bulls the presence of low molecular weight (LMW 13KDa and 33KDa) proteins was associated with low motility (35-40%) in accordance with Chacur et al. (3). There was a significant increase ($P < 0.05$) in major spermatic defects in these six bulls (20.3 ± 3.7%) associated with presence of proteins that had molecular weights of (23, 35 and 72KDa). In six Brown-Swiss and eight Brangus bulls, 55KDa, 66KDa or 80KDa proteins were present and associated with a satisfactory semen condition (motility 77 ± 6% and major defects 5 ± 2%) in accordance with Chacur et al. (3). In cattle, the 55, 66 and 80KDa proteins are associated positively with camp-dependent progressive motility (1). Consistently, in the present experiment, there was a positive relationship of presence of seminal plasma proteins 55KDa, 66KDa and 80KDa and semen quality (motility and major defects). The presence of these proteins suggests an increase in semen quality.

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Testicular degeneration induced by electromagnetic fields in rats: light and electron microscopy study

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Introduction

Generation, distribution and use of electric energy can generate low frequency electromagnetic fields (EMF), however, the effects of EMF on spermatogenesis are not entirely clear. The present study aims to investigate the EMF interaction in spermatogenesis of rats during different periods of testicular development.

Materials and Methods

Pregnant Wistar rats (*Rattus norvegicus, var. Albinos*) were separated into two groups, a control (n=5) and exposed to EMF from the 13th day of gestation (n = 10). After birth, male pups were randomly chosen and continued to be exposed to EMF up to 90 postnatal days (n = 12); or exposed to EMF up to 21 postnatal days and analyzed at 90 postnatal days (n = 12); or control male rats (n = 12). The treated groups were placed in a cylindrical compartment of PVC centrally located in Helmholtz coils (PHYWE - GERMANY), emitting EMF with frequency of 60 Hz and magnetic flux intensity of 1 mT. The control group underwent the same experimental conditions except for the presence of EMF. Testicular fragments were embedded in plastic resin of glycol methacrylate (Leica), cut with 4 µm thick, stained with 1% toluidine blue / sodium borate and morphologically analyzed. Testis fragments were routinely processed to electron microscopy and analysis was performed by a FEI Morgani 268D transmission electron microscope.

Results and Discussion

No pathological changes were observed in control animals. The results showed testicular degeneration in animals exposed to EMF, both up to 21 and 90 postnatal days. This condition in the testicle was more severe in animals exposed to EMF up to 90 postnatal days. Optical microscopic analysis showed loosening seminiferous epithelium, sloughing of germ cells, elongated spermatids with tail swelling, intertubular space enlarged, thickening of tunica propria, Sertoli cells vacuolation, as well as large amount of germ cells in necrosis and apoptosis. Ultrastructural analysis of the testis showed several Sertoli cells with intracitoplasmatic vacuolation and mitochondria with increased electrondensity and loss of mitochondrial cristae in germ cells. Previously, it was observed that electric and magnetic fields can induce a reduction in the counting of sperm number, motility and daily sperm production in rats, as well as adverse effect on sperm quality in human (1, 2). Therefore, the testicular degeneration and reduction of mitochondrial function related to 60 Hz and 1 mT EMF exposure may result in subfertility and infertility.

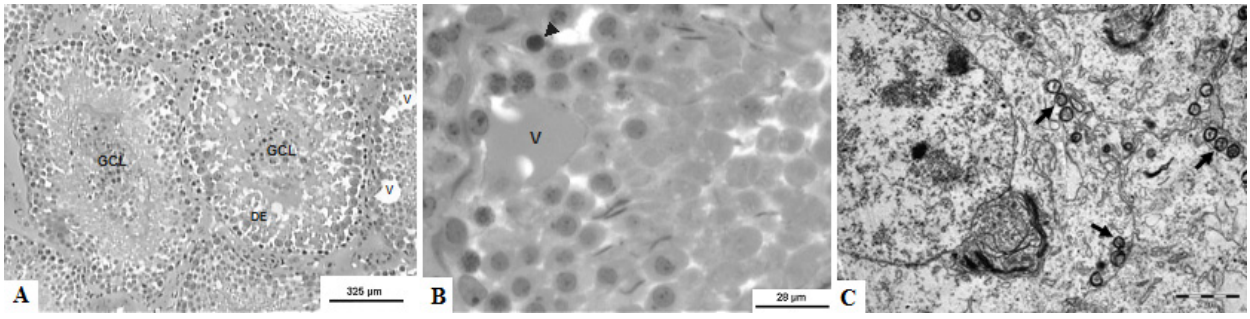

Figure 1. Photomicrograph and electronphotomicrograph of testis in rats exposed to EMF from gestation up to 21 (Fig. 1A and 1C) or 90 postnatal days (Fig. 1B). In Fig. 1A can be observed desquamation of seminiferous epithelium (DE), desquamated germ cells in tubular lumen (GCL) and vacuolization in Sertoli cells (V). Note in Fig. 1B vacuolization of Sertoli cell (V) and a germ cell in pyknosis (arrowhead). Figure 1C shows round spermatids with high electrondense mitochondria and loss of their cristae (arrow).

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Electrophoretic characterization of Morada Nova rams seminal plasma heparin-binding proteins

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Introduction

Morada Nova is an important ovine breed to the economy of Brazilian Northeastern, due to its adaptability to semi-arid conditions and also for the good leather quality. However, limited information about its reproductive efficiency especially of rams impairs higher advances in productivity of that breed. Heparin-binding proteins are the major proteins present in seminal plasma and play important roles on sperm reproductive function, such as capacitation, acrosome reaction and sperm-egg fusion. Their quantitation, identification and biochemical properties are well established in many species, but information about their function on reproductive physiology of Morada Nova rams are scarce. Thus, the aim of this study was to characterize by 1-D electrophoresis the heparin-binding proteins of Morada Nova seminal plasma, pre-purified by heparin affinity chromatography.

Material and Methods

Seminal plasma samples were obtained by centrifugations of raw semen from 4 Morada Nova rams. Aliquots of 300 µL of seminal plasma were subjected to heparin affinity chromatography, with an initial flux of 0.5 mL/min of binding buffer (Tris 40 mM, $CaCl_2$ 2 mM, Sodium Azide 0.01%, PMSF 1 mM). After 5 minutes, flux was accelerated to 2 mL/min and at 30 minutes the elution buffer (binding buffer plus NaCl 1M) was applied to unbind the retained proteins, which were pooled, desalted and freeze-dried. Chromatograms were plotted by evaluation of fractions absorbance at 280 nm. Heparin-binding proteins were diluted in 300 µL of Milli-Q water for total protein quantitation (1). Five micrograms of ram seminal plasma heparin binding proteins were subjected to one-dimensional electrophoresis (SDS-PAGE) to determination of proteins mass according to Martins et al. (2). Gel was silver stained and scanned for computational evaluation using Quantity One Software, v.4.6.3.

Results and Discussion

Figure 1A shows the two peaks from heparin affinity chromatography. The elution time of 30 minutes with binding buffer was sufficient for total exclusion of non-binding proteins, before the application of elution buffer, guarantying good separation of heparin-binding proteins. They corresponded to 25.4% of whole seminal plasma. The 1D gel reveals the complexity of seminal plasma heparin-binding proteins where, in average, 37 bands were detected by silver staining, varying from 13.3 to 229.6kDa. The most expressed heparin-binding proteins are displayed by arrows in figure 1B. The band of 50.45 kDa is close to the molecular weight (MW) of Osteopontin (55kDa) which contains 2 heparin binding domains (3). Band 21.57kDa has similar MW of RSVP22, a BSP-like protein found in ram seminal vesicle fluid. The 14.08kDa band similar MW of RSVP14, another BSP-like protein, and also a spermadhesin-like protein called Bodhesin. The heparin binding proteins are very important for several sperm functions, thus the purification of them may provide further studies to understand their role in Morada Nova ram reproductive physiology.

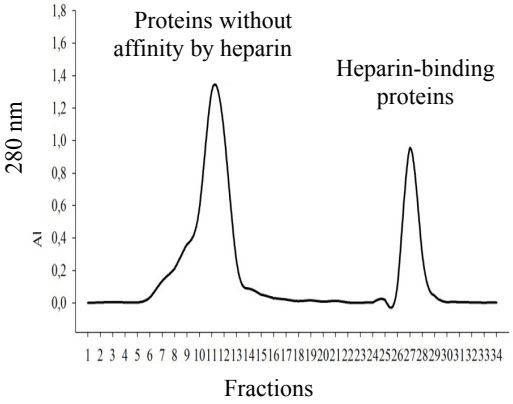
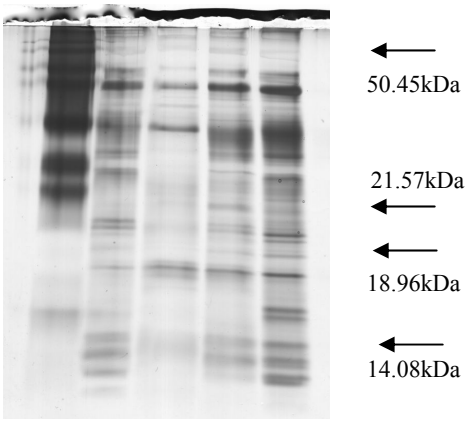


Figure 1. A – Heparin Affinity chromatogram of Morada Nova rams seminal plasma.



B – 1D Gel of Heparin affinity proteins of Morada Nova rams seminal plasma.

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The collared peccary (*Tayassu tajacu*) as a new experimental model to investigate the spermatogonial stem cells niche

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Introduction

Several studies demonstrated that spermatogonial stem cells (SSCs) are preferentially located in a special microenvironment called "niche", and this microenvironment is probably provided by the somatic Sertoli and peritubular myoid cells, the basement membrane, and other cellular components from the interstitial compartment. Recently, Yoshida et al. (1) reported the influence of the vascular network in the spermatogonial distribution in the seminiferous tubules but the true role of Leydig cells in the SSCs niche is not yet clearly elucidated. Previous studies developed in our laboratory showed that peccaries have a peculiar Leydig cell organization and distribution around the seminiferous tubules lobes (2). Due to this particular cytoarchitecture, peccaries represent an interesting model for investigating SSCs niche. Thus, the present study aimed to morphologically characterize the different types of spermatogonial cells and evaluate their location/distribution in the peccary testis parenchyma.

Material and Methods

Testis from eight adult collared peccaries (*Tayassu tajacu*) was fixed in 4% glutaraldehyde and routinely prepared and embedded in plastic for histological and stereological analyses. Characterization of spermatogonial cells was performed based on the pattern of heterochromatin distribution (3), the nuclear volume, and localization of these cells in the seminiferous epithelium. To determine the spermatogonial location, three different regions in the testis parenchyma were used as a reference point: Tubule-Tubule (T-T), Tubule-Interstitium (T-I) and Tubule-Leydig Cells contact (T-LC). The density of spermatogonial cells per each region was evaluated according to the number of spermatogonia per area expressed in degrees, considering that the entire seminiferous tubule circumference is 360^{o}.

Results and Discussion

Seven different types of spermatogonial cells were clearly distinguished in this species: A undifferentiated (A_{und}), A_1, A_2, A_3, A_4, In, and B. Compared to the other spermatogonial cell types, A_{und} spermatogonia in peccaries are very peculiar because they have a noticeably higher nuclear volume ($p < 0.05$; Fig.1). This particular characteristic allows more precisely the evaluation of the distribution of A_{und} along the seminiferous epithelium. These cells were observed in all ten stages of the seminiferous epithelium cycle characterized for this species and presented a preferential location for T-I region ($p < 0.05$; Fig.2). This result corroborates the data already observed for other mammals, demonstrating a non-random distribution of the A_{und} in the seminiferous tubule. Noticeably, this finding strongly suggests that Leydig cells play a negative role in the A_{und} niche. In order to better understand the SSC biology and niche in mammals, we are currently developing studies aiming to find potential or specific markers for peccaries SSCs.

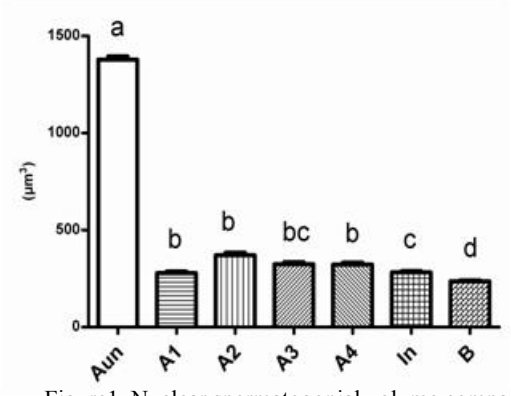

Figure1. Nuclear spermatogonial volume compared.

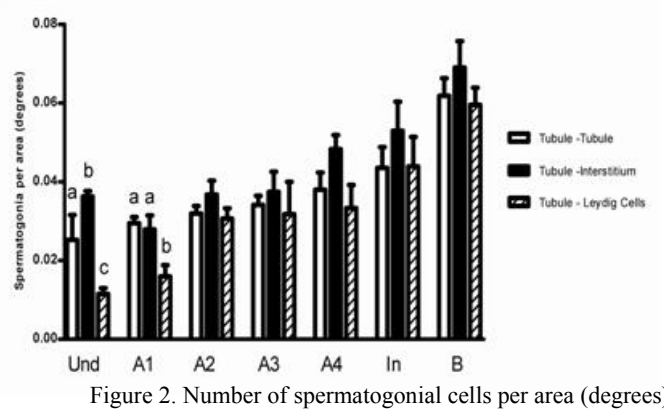

Figure 2. Number of spermatogonial cells per area (degrees).

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LIF affects the kinetics of bovine type A spermatogonia *in vitro*

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Introduction

Spermatogonial stem cells (SSC's) are a subset of type A spermatogonia capable of self-renewal in the testis. LIF (Leukemia inhibitory factor) is an important growth factor which is required for inducing mouse SSC's to become reprogrammed into pluripotential embryonic stem cells (1). We tested the effect of LIF on bovine germ cell cultures including somatic cells with predominance of Sertoli cells.

Materials and Methods

Testes of 6 month old calves were collected from commercial slaughterhouses and germ cells isolated and cultured for a maximum of 21 days. General proliferation was evaluated with WST1 assay at various time points and LIF concentrations (0, 1, 10, 100 ng/ml). Bovine type A spermatogonia were marked with Dolichos biflorus agglutinin (DBA) lectin and proliferation was evaluated through Proliferating cell nuclear antigen (PCNA) immunohistochemistry.

Results and Discussion

A level of LIF of 1ng/ml resulted in significantly higher values of proliferation at days 16 and 21 ($P < 0.01$). Germ cell colonies appeared during culture, showing no significant difference in numbers in control and LIF treated cultures ($P > 0.05$). Type A spermatogonia (DBA+) were mostly physically associated with colonies and were significantly higher in numbers in LIF cultures at day 7 ($P < 0.05$). Proliferation (PCNA+ cells) of both type A and somatic cells was similar in control and LIF cultures. The increase of type A spermatogonial numbers in the presence of LIF can probably be explained in terms of increased survival rather than an increase in proliferation. It will be very important in the future to test the stem cell function and embryonic germ layer tissue derivation of bovine type A spermatogonia under the influence of LIF.

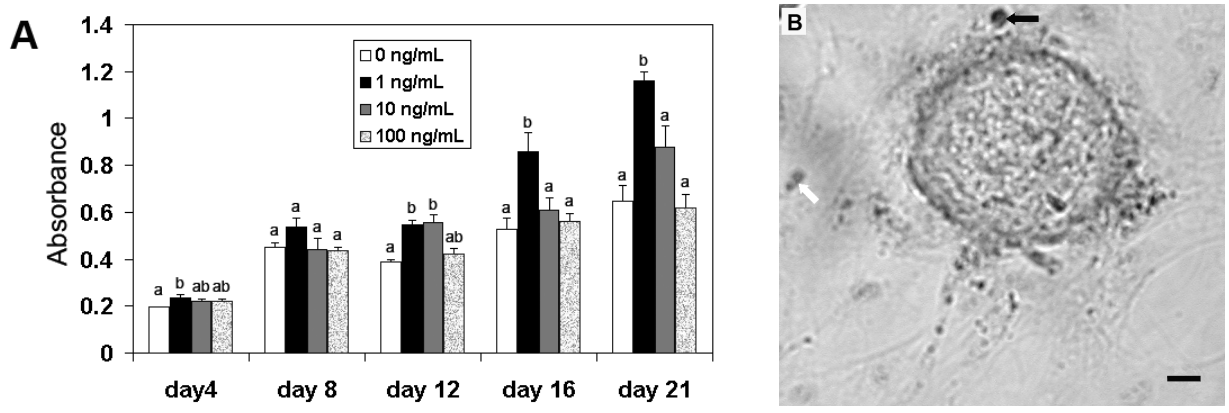

Figure 1. A) General proliferation of 21 days of duration germ cell cultures evaluated with WST1 assay at various LIF concentrations. Different superscript letters represent significant differences ($P < 0.01$). B) Proliferating cells marked with PCNA in bovine germ cell cultures. Black arrow: PCNA positive type A spermatogonia adjacent to a colony. White arrow: PCNA positive somatic cell, most likely a Sertoli cell. Bar = 25 μm.

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Seasonal effect on semen quality in teratospermic domestic cats in the northern hemisphere

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Introduction

Photoperiod and temperature are important factors that may influence the reproductive cycle by regulation of pituitary and gonadal functions (1, 4). The effect of day length on breeding seasonality seems to disappear below 30º of latitude (2). The objective of this study was to investigate the seasonal influence in seminal characteristics in cats living in identical conditions in northern hemisphere, in the north of France

Materials and Methods

Five adult Siamese-related cats, average aged 3.6 years and average weight 4.8kg were used in the study. The cats were housed in the same cattery throughout the period of the study (December 2009 -May 2010). The semen was collected by eletroejaculation with stimulation protocols used for Tebet et al. (3), and the anaesthetic protocol used was the association, IM, 5 mg/kg of ketamine (Imalgene 1000®) plus 0.04 mg/kg of medetomidine (Domitor®). Immediately after collection, spermatic progressive motility, progressive velocity, ejaculate concentration, integrity of membranes, spermatic abnormalities (4) of the acrosomes, midpieces, sperm tails, and head defects were evaluated. The period was divided into 3 seasons: I: winter (December, January and February), II: transitional period (March) and III: spring (April and May). Differences between seminal characteristics (median ± SD) were determined by ANOVA followed by a Kruskal-Wallis test. Values were considered significant at $p \leq 0.05$.

Results and Conclusion

All cats were teratospermic during the whole experiment; median values of semen characteristics, during seasons (I, II and III) were, respectively: sperm motility (80; 85; 70%), sperm velocity (4.5; 4.0; 4.0), total concentration (75.5; 34; 68 ($x10^6$ spermatozoa)), normal sperm (35.5; 28; 32%). The most frequent spermatic alterations were acrosome defects (11; 13; 17.3%), head defects (2; 3; 4%), intermediary piece (19; 28; 25.5%), tails defects (25; 21; 24.5%). We observed an important (but not significant) increase in the acrosomal defects ($p = 0.0551$) and head defects ($p = 0.054$) in spring. The membrane integrity was however not altered during this period. 4/5 cats were used for artificial insemination with this period and were proved to be fertile. The main difference with other studies may be that these cats were teratospermic which may induce differences with what is observed in normospermic cats. In conclusion, there seems to be only moderate seasonal effect on semen quality in teratospermic cats in the northern hemisphere.

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Somatic index of different testicular components in dogs from puberty to senility

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Introduction

The dog presents more than 400 recognized breeds, being the mammallian species with the largest variability of corporal mass. Somatic Index (ratio of corporal mass allocated in different testicular structures) might be used to compare individuals of different ages and distinct corporal mass (1). In addition to the corporal size, the Testicular Somatic Index is also under the influence of other factors such as age and mating system (2). This study aimed to define the somatic index of different testicular components in dogs from puberty to senility.

Material and Methods

Fifty-six mixed breed dogs were weighed, and had their testis removed and weighed with and without the albuginea. A fragment of each testis, of approximately 0.1 cm^3, was fixed in buffered formalin and processed for histological examination. The volumetric proportion of the components of the testicular parenchyma was measured observing the histological sections under a light microscope, equipped with integrating eyepiece containing 475 points. It was observed, for each animal, 20 fields randomly distributed. The overall volumes of seminiferous tubules and Leydig cells were estimated by inferring the volumetric proportion of each component from the volume of the testicular parenchyma. Somatic index were obtained from the testicular weight, testicular parenchyma weight, overall volumes of seminiferous tubules, Leydig cells volumes and testicular albuginea weight.

Results and Discussion

It was observed a tendency in the increase of the Testicular Somatic Index until the 6 years of age, when then the values declined in the animals 10 years old or more (Table 1). The Testicular Somatic Index had a major contribution from the testicular parenchyma than from the testicular albuginea in different ages. The Seminiferous Tubule Somatic Index increased until the age of 5 to 6 years, decreasing to minimum values in the animals 10 years old or more. The Leydig Cell Somatic Index followed the age-related observations registered for the Seminiferous Tubule Somatic Index.

Table 1. Body weight, testicular weight, Testicular Somatic Index, Testicular Parenchyma Somatic Index, Testicular Albuginea Somatic Index, Seminiferous Tubule Somatic Index, Leydig Cell Somatic Index in dogs from puberty to senility.

Age (years)	Body Weight (kg)	Testicular Weight (g)	Testicular Somatic Index (%)	Testicular Parenchyma Somatic Index (%)	Testicular Albuginea Somatic Index (%)	Seminiferous Tubule Somatic Index (%)	Leydig Cell Somatic Index (%*10^3)
1-2	11.25 ± 4.03	13.91 ± 5.61	0.10 ± 0.01^{AB}	0.074 ± 0.01^{C}	0.019 ± 0.002^{A}	0.07 ± 0.01^{AB}	3.11 ± 0.99^{AB}
3-4	15.70 ± 3.46	17.15 ± 6.64	0.12 ± 0.02^{AB}	0.098 ± 0.02^{B}	0.019 ± 0.004^{A}	0.09 ± 0.03^{BC}	4.55 ± 1.59^{BC}
5-6	13.00 ± 4.19	17.12 ± 7.40	0.14 ± 0.05^{A}	0.15 ± 0.03^{A}	0.027 ± 0.005^{B}	0.11 ± 0.04^{C}	5.84 ± 2.1^{C}
7-9	19.87 ± 10.22	20.33 ± 10.13	0.10 ± 0.02^{AB}	0.086 ± 0.02^{ABC}	0.018 ± 0.004^{A}	0.08 ± 0.01^{BC}	5.27 ± 2.51^{BC}
10<	23.12 ± 8.32	18.80 ± 6.24	0.08 ± 0.02^{B}	0.07 ± 0.01^{C}	0.015 ± 0.005^{A}	0.06 ± 0.01^{A}	2.49 ± 0.99^{A}

^{AB}Means with different letters in the same column differ statistically ($P < 0.05$).

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Delta-like homolog I (Dlk1) is a novel marker for prepubertal Sertoli cells

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Introduction

Spermatogenesis is one of the most efficient cell-producing systems in adult animals. At the origin of this process are spermatogonial stem cells (SSCs), which have the potential to self-renew and differentiate to ultimately form spermatozoa. Somatic Sertoli cells and germ cells are present in the seminiferous epithelium where, by interacting physically and biochemically, they create an ideal microenvironment for germ cell development, survival and differentiation (1). One potential signaling pathway involved in cell fate decision during spermatogenesis is the Notch pathway (2, 3). The protein Delta-like homolog I (Dlk1) is a member of the Epidermal Growth Factor (EGF) family that includes Notch receptors and their ligands (Delta, Delta-like (Dll), and Jagged), which are involved in cell signaling and cell fate determination (4). Because the membrane-bound and soluble forms of Dlk1 are both biologically active, Dlk1/Dlk1 interactions are possible, suggesting that Dlk1 might act in a justacrine and paracrine manner, which is fundamental for cell communication. Dlk1 is expressed in multiple embryonic tissues but is not detected after birth, except in cell types involved in proliferation and/or differentiation. Dlk1-knockout mice present with growth retardation, obesity, blepharophimosis, skeletal malformation, and increased serum lipid metabolites. About 50% of *dlk1* homozygote null mice die two days after birth, demonstrating the importance of this molecule for animal growth and survival (5). In the present study we investigated for the first time the Dlk1 expression in the seminiferous epithelium at different ages after birth.

Material and Methods

Testis from 0, 4, 6, 8, 16, and 44 days-old-mice were collected, fixed in paraformaldehyde and processed for paraffin embedding. Tissue sections (5 µm-thick) were processed for immunohistochemistry and immunofluorescence to detect Dlk1 expression. Total RNA samples extracted from testicular tissues and Sertoli cells were used for Real-Time PCR analysis of gene expression. *Dlk1*, *dazl*, and *vim* probes were utilized. The expression value of *dlk1* mRNA was normalized to the expression of an internal control gene (18s ribosomal RNA) to calculate the relative amount of RNA in each sample.

Results and Discussion

We found for the first time that Dlk1 is present in the mouse testis, and is a novel molecular marker specifically expressed by Sertoli cells shortly after birth (day 0-4). It is significantly down-regulated afterwards. These results suggest that Dlk1 might be involved in a signaling pathway affecting gonocyte migration or differentiation into SSCs. Another hypothesis is that Dlk1 might be important for maintenance of the undifferentiated state of Sertoli cells. Future functional studies will determine the role of Dlk1 in the first steps of spermatogenesis. Identification of molecular markers and signaling pathways involved in somatic cell/germ cell interactions will help advance infertility treatments, genetic manipulations, transgenesis, contraception, and other potential applications.

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Stereological investigation of the adult nude mice testis

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Introduction

The development of new technologies to produce mutant animals provides an excellent approach for better understanding the physiological processes, including reproductive biology. The homozygous mice for the mutation *nude* (1) are athymic and therefore very attractive immunodeficient experimental models. For instance, it is already known that athymic male mice have reduced gonadotropins (LH and FSH) and testosterone in comparison to their normal littermates (2). In this regard, as these hormones are very important for the testis development and function, we are currently performing comparative studies in order to investigate several key parameters related to spermatogenesis in the Nude mice, including the estimation of seminiferous epithelium cycle length.

Materials and Methods

Nine Nude and eight wild type Balb C male mice were utilized. The testis were dissected, weighed and fixed with 4% buffered glutaraldehyde. Testis tissue fragments were embedded in plastic (glycol methacrylate) and routinely processed for histological and morphometric analyses. Before sacrifice, intraperitonial injections of tritiated thymidine were performed at two different time periods (1 hour and 9 days) in order to estimate the duration of spermatogenesis. The different stages of the seminiferous epithelium cycle were characterized according to the acrossomic system method and their frequencies were estimated from the analysis of at least 250 seminiferous tubules cross-sections per each mouse. All data are presented as the mean ± SEM. Student t-test was performed using GraphPad Prism 5.00.288 and the signicance level was set as $P < 0.05$.

Results and Discussion

Compared to their controls (Balb C), the Nude mice had a significantly lower ($p < 0.05$) testis weight (83 vs. 103 mg) and the same trend was observed for the gonadosomatic index (0.59 vs. 0.77%) that is the total testis mass divided by the body weight. Twelve stages of the cycle were characterized. Except for the stages IV and XI, the results found for stage frequencies were different in the two mice strains investigated (Fig. 1). Particularly, the frequencies of stages I, VII and XII were significantly higher in Balb C, whereas stages II/III, V, VI, VIII, IX and X presented higher frequencies in Nude mice. Nevertheless, based on the stages frequencies and the most advanced germ cell type labeled after the two tritiated thymidine injections, the total duration of spermatogenesis in the Nude (39.6 days) and the Balb C mice (40.1 days) was very similar. When compared to the Balb C, the preliminary morphometric data obtained showed that the tubular diameter was higher in Nude mice (246 ± 6 vs. 210 ± 3μm). We are currently performing a more detailed morphometric analysis in these mice, in order to quantify Sertoli cells and spermatogenic efficiencies, as well as other important Leydig cells parameters.

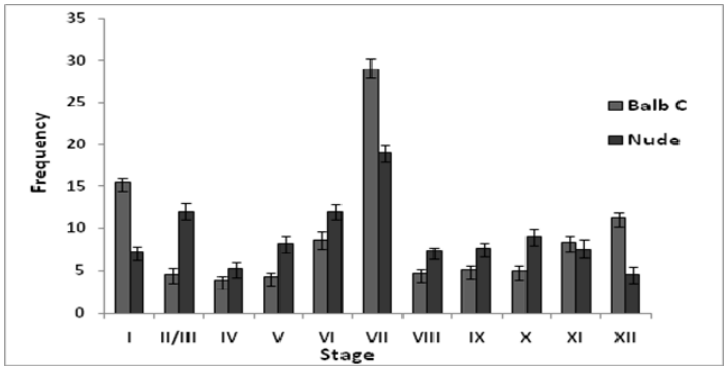

Figure 1. Stage frequencies (%) in Nude and Balb C mice. These stages were characterized according to the develop-ment of the acrossome.

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Obesity increases FGF content and led to stromal hypertrophy and premalignant lesions on the rat ventral prostate

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Introduction

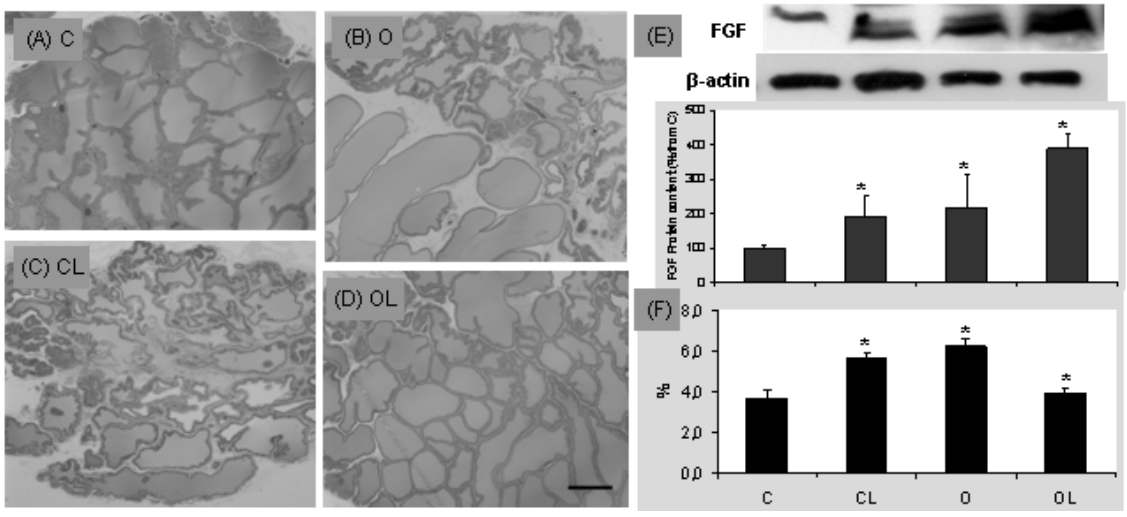
Obesity increases estrogen (E) levels due a higher conversion of testosterone (T) by aromatase and also decreases circulating T. Although both hormones are crucial for prostate homeostasis, the effect of obesity on this organ remains unknown. This study evaluates the effect of obesity induced by high fat diet on histology of the rat ventral prostate (VP). The influence of E in such putative alterations is also evaluated by means of treatment with aromatase inhibitor Letrozole.

Material and Methods

Four groups of adult male Wistar rats were employed: control (C), control + Letrozole (CL), obese (O) and obese + Letrozole (OL). Obesity was induced by treatment with diet containing 20% of satured fat for 15 weeks. Letrozole (1 mg/kg b.w/day) was administered orally for 2 weeks; C and O groups received the vehicle only. The diet was maintained during treatments. Rats were killed, biometrical and metabolic parameters were determined and the VP was processed for immunohistochemical, transmission electron microscopy and Western blotting analyzes.

Results and Discussion

Obesity led to a 70% decrease in plasma T, insulin resistance and hyperinsulinemia, but did not affect the E levels. It caused cystic atrophy in the intermediate region of VP and epithelial shrinkage in the distal region (Fig. A, B). Prostatic intraepithelial neoplasia and inflammation were more frequent in O in comparison with other groups. T and E were unchanged in CL and OL when compared with C. The VP of CL rats exhibited atypical atrophyc acini and that of OL was histologically similar to C (Fig. C, D). The smooth muscle cells (smc) increased in CL and O and did not change in OL prostate (Fig. F). Ultrastructural analysis indicated that Letrozole caused disorganization of smc cytoskeleton in both CL and OL. Obesity increased 150% the prostatic content of FGF-2 and Letrozole per se also increased this protein (Fig. E).



Figures. (A-D) Histology of ventral prostate. (E) Western blot of FGF-2 (upper) and quantification (low). (F) Relative frequency of smc. Legend: C-control, CL-control plus Letrozole, O- obese, OL-obese plus Letrozole. The values in E and F represent Mean ± S.E.M. * Significant difference compared to respective control ($p \leq 0.05$).

Obesity affects the structure of rat prostate, increasing the incidence of pathological lesions. The role of E in such alterations could not be clarified but Letrozole treatment restores them partially. The smc hipertrophy and increased FGF-2 expression resulting from obesity can be linked to a further development of benign prostatic hyperplasia.

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Effects of Inducible Nitric Oxide Synthase (iNOS) deficience on postnatal testis development in mice

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Introduction

Nitric oxide (NO) is an important mediator of intra and extra cellular processes and has been shown to play a crucial role in several physiological and pathological conditions. Nitric oxide synthase (NOS) produces NO via catalysis of L-arginine in mammalian tissues. Various isoforms of NOS are expressed in the testis, such as neuronal (nNOS), endothelial (eNOS) and inducible (iNOS), and all of them seem to be important for the normal function of this tissue (1). Particularly, the iNOS isoform produces high levels of NO independent of intracellular calcium (2) and is found in Sertoli, Leydig, and germ cells, including spermatozoa (3). Therefore, studies involving the role of NO and iNOS are important for reproductive biology and the better comprehension of testis function, potentially providing treatments for male reproductive disfunctions. In this regard, the aims of this investigation were to study the effects of iNOS deficience during prepubertal postnatal testis development in mice, using quantitative and qualitative approaches.

Materials and Methods

Twelve wild type C57BL/6 and ten knockout iNOS male mice were utilized and distributed in 3 groups: 01, 10 and 20 days of age. Testes were immersion-fixed (01 day) or perfused-fixed (10 and 20 days) with 4% glutaraldehyde, 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 GraphPad Prism 5.00.288. $P < 0.05$ was considered significant.

Results and Discussion

The body weight in KO mice was higher ($P < 0.05$) at 01 and 10 days of age, and in all ages investigated the testis weight was significantly increased (from ~50 to ~90%). Therefore, the gonadosomatic index (testes mass divided by body weight) followed the same tendency observed for the testis weight. At 01 day, the volume density of seminiferous tubules in iNOS-deficient mice was greater ($P < 0.05$), whereas the opposite trend was observed for the intertubular compartment volume density in these mice. However, no significant difference was observed for Leydig cell volume density. In comparison to the wild type, at 01 and 10 days of age, the diameter of the seminiferous cords/tubules was higher ($P < 0.05$) in iNOS-deficient mice. As it is known from the literature this parameter present an excellent correlation with the evolution of spermatogenesis during postnatal testis development, reflecting also the degree of Sertoli cell maturation and fluid secretion that ultimately result in lumen formation. Although no differences were observed at the other ages investigated, at 01 day of age the Sertoli cell nuclear volume was significantly decreased (~25%) in iNOS-deficient mice, and the lumen was observed for the first time in 10 days old KO mice. Taken together, these data already obtained suggests that inducible nitric oxide synthase (iNOS) plays an important role in testis function during postnatal development and probably also in fetal life. Therefore, we are currently investigating other representative ages, including the period after testis differentiation that might be critical for the better understanding of the effects of this enzyme in male reproduction.

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Influence of progesterone and its interactions with estrogen on the Mongolian Gerbil (*Meriones unguiculatus*) male prostate

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Introduction

The physiological role of androgen in the prostate regulation is already well known, as well as the regulatory effects of estrogen on androgen action, but little is known about association of these with the progesterone hormone. The expression of progesterone receptors in prostatic tissue showing benign and malignant lesions has been reported by some studies (1, 2). Thus, this study aims to evaluate the effects of progesterone and its interactions with estrogen on the prostate tissue in male gerbil (*Meriones unguiculatus*).

Materials and methods

Mongolian gerbils, with 45 days of age, underwent bilateral orchiectomy. At 90 days of age animals were divided into 4 groups: normal control (NC) - not castrated; castrated control (CC) – castrated gerbils; castrated plus progesterone (CaP) and castrated plus progesterone and estrogen (CaPE) - castrated animals that received subcutaneous dose of progesterone and estrogen. All treated animals received subcutaneous doses of hormones for 14 days every 48 hours. After treatment the animals were sacrificed, blood was collected and the complex prostate removed. Analyses of the general morphology were conducted by stereology and morphometry, as well as immunohistochemistry.

Results and discussion

With castration, a reduction of prostatic alveoli and a significant decrease in testosterone levels (2.13 ± 0.29 to 0.02 ± 0.01 ng/ml) were observed. The epithelium became folded with smaller secretory cells (19.6 ± 0.23 to 11.79 ± 0.28 μm). The stromal compartment became disorganized, containing a thinner and irregular muscular layer (14.11 ± 0.33 to 11.87 ± 0.22 μm). A reduction in the intensity of secretion was also observed. The expression of androgen receptor (AR) migrated from the epithelium to the stroma and there was an increase in cell proliferation in the smooth muscle layer. In the CaP group there was a significant increase in the size of secretory cells (19.6 ± 0.23 to 14.62 ± 0.19 μm), estrogen levels (23.16 ± 4.22 to 53.44 ± 29 pg/ml), and little change in alveolar size. AR became more evident in the stroma, as well as cell proliferation. Across the CaPE group there was a maximum increase of epithelial cell size (19.6 ± 0.23 to 24.86 ± 0.29 μm), alveoli prostatic and also in the estrogen (23.16 ± 4.22 to 119.06 ± 31 pg/ml) and progesterone levels (0.48 ± 0.09 to 20.14 ± 9,99 ng/ml). The stromal layer become more organized, formed by thickened muscle cells (14.11 ± 0.33 to 17.46 ± 0.38). In this treatment, the AR was again expressed in prostatic epithelium and an increased proliferation in the same prostatic compartment was observed. The administration of progesterone provided an increase in prostatic secretion, which was intensified by the addition of estrogen. The replacement of these hormones showed potential to restore the structure and secretory activity of the prostate gland. Regarding morphological restoration of the prostate, however, none of these hormones were potentially capable of reversing to normal glandular structure. The differential expression of androgen receptors and cell proliferation markers, given the different treatments, shows the importance of hormonal homeostasis to the balance of prostate tissue morphophysiology. Thus, it is important to better understand the effects that key reproductive hormones have on the development and regulation of the prostate gland.

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Ovarian function after induction of ovulation and gonadotrophic supplementation of overconditioned Nelore cows (*Bos taurus indicus*)

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Introduction

Nutrition affects reproduction in postpartum beef cows. There are consistent evidences that overconditioning of cows is detrimental to reproduction (1). The present study evaluated ovarian function in postpartum Nelore (*Bos taurus indicus*) beef cows with different body condition scores (BCS) treated with luteal function enhancers after induction of ovulation.

Material and Methods

Twenty non-suckled cows (body weight = 452.7 ± 54.9 kg and 121.0 ± 33.2 days postpartum) received an auricular implant (3mg norgestomet - Crestar, Intervet) and an IM injection of 2mg of estradiol benzoate. Nine days later, implant was withdrawn and 2mg of estradiol benzoate (IM) were given after 24 hours. Eleven cows had their ovulation confirmed (day zero) through ultrasound and remained in the trial. Females were split into two groups according to their BCS (1-9 scale; 2), as follows: normally conditioned cows group ($G_{BCS=5.4}$; n = 6) and overconditioned cows group ($G_{BCS=6.7}$; n = 5). All cows received a GnRH agonist (IM; 200µg of gonadorrelin - FERTAGYL®, Intervet) on day 5 (D_5) and 2500 IU of hCG (IM; CHORULON 5000 UI®, Intervet) on D_{12}. Ultrasound examination of ovaries and blood sampling were carried out 48 hours after implant removal until the subsequent ovulation on a daily basis and observation of estrus behavior was conducted twice a day. Blood plasma was obtained and progesterone concentration ([P_4]) was determined through a validated RIA. Results were analyzed by ANOVA.

Results and Discussion

The dominant follicle of the third wave in overconditioned cows persisted longer ($P < 0.05$) than $G_{BCS=5.4}$ cows. Estrous cycle length, luteal phase length, number of waves of follicular development, number of corpora lutea, maximum [P_4] within the estrous cycle, interovulatory interval were not affected by BCS. On the other hand, daily means of [P_4] throughout the luteal phase of estrous cycle was greater ($P < 0.05$) for $G_{BCS=5.4}$(5.97 ± 1.3 ng/ml) as compared to overconditioned cows (4.87 ± 1.5 ng/ml), which also showed smaller ($P < 0.05$) maximum volume of corpus luteum (10,568 ± 3,764 mm^3) than $G_{BCS=5.4}$ cows (16,492 ± 3,764 mm^3). In addition, the diameter of the preovulatory follicle differed ($P < 0.05$) according to BCS (13,1 ± 1,8 mm to $G_{BCS=5.4}$ cows and 11,1 ± 3,8 mm to $G_{BCS=6.7}$ cows). In conclusion, ovarian function was influenced by BCS in a way that corpus luteum development, P_4 secretion and final growth of the preovulatory follicle were depressed in overconditioned Nelore cows submitted to hormone treatments to induce ovulation and enhance subsequent luteal function. In a previous report (3), cows with higher BCS presented greater [P4]. However, the same cows also promoted altered follicular dynamics after induction of ovulation. It is speculated that steroids synthesis or degradation in overweight cows is somehow altered due to abnormal fat metabolism. As a result, reproductive cyclic events may be negatively affected in overweight cows.

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Characterization of MAS receptor and ACE2 expression in granulosa cells of bovine preovulatory follicles

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Introduction

The renin angiotensin system (RAS) has important roles on mammalian reproductive function including the ovulatory process. Expression of Ang1-7 receptor MAS and angiotensin-converting enzyme 2 (ACE2) is increased in ovary of rats treated with eCG (1). However, the function of Angiotensin 1-7 (Ang1-7) is not well understood in ovaries of monoovulatory species. The objective of this study was to determine the expression of MAS receptor and ACE2 in different cellular types of preovulatory follicles in cattle. In addition, we investigated the expression of MAS and ACE2 before and after the LH surge during the ovulatory process.

Materials and Methods

Forty cows were synchronized and those with follicular diameter ≥ 12 mm were ovariectomized at different periods (0, 3, 6, 12 or 24 h) after GnRH injection. Theca and granulosa cells were removed from the follicle (2) and submitted to RNA extraction to evaluate the relative expression of the genes MAS and ACE2 using Real-time RT-PCR assay. The results of gene expression were compared by analysis of variance (PROC GLM). When treatment effects were observed, the means between the different groups were compared using multi-test comparison of means (LSMEANS).

Results and Discussion

The expression of MAS and ACE2 was detected in theca and granulosa cells. In theca cells no differences on expression of MAS and ACE2 (Fig. 1A) were detected at different times after treatment with GnRH. In granulosa cells, there was a greater expression in the early hours (0 h) and end (24 h). Near ovulation (2 4h after GnRH), ACE2 expression was significantly up-regulated compared to 12h after treatment ($P < 0.05$) (Fig. 1B). The results showed greater expression of the enzyme responsible for cleavage of Ang II. In another study, an increase in the concentrations of ANG II was observed 24 hours after the LH peak (3). Taken together, these results indicate a role of Ang(1-7) in the ovulatory process in cattle.

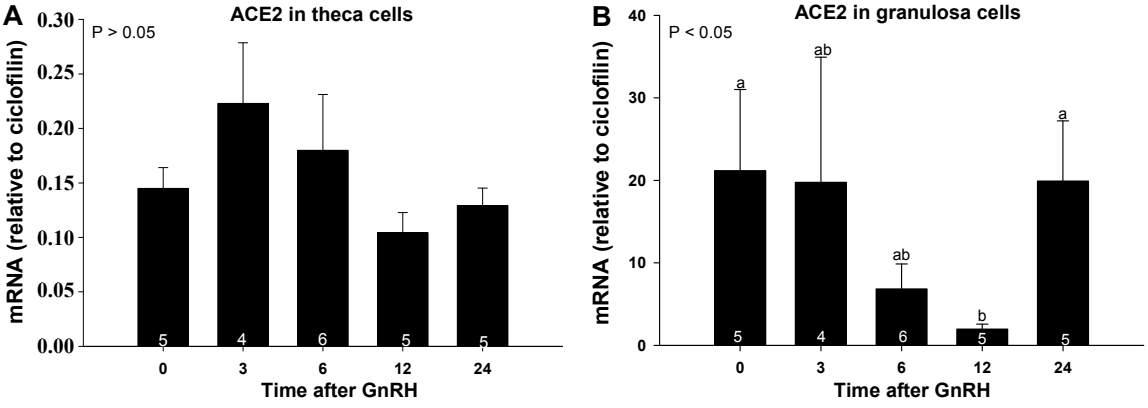

Figure 1. Relative mRNA expression (mean ± standard error of mean) of the angiotensin-converting enzyme 2 (ACE2) in the theca cells (A) and in granulosa cells (B), at different times after application of GnRH. Values in bars represent the total number of animals (follicles) per group. Different letters represent statistical difference ($P < 0.05$).

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Expression of Grb10 and Grb14 mRNA in bovine cumulus-oocyte complexes from follicles at different stages of development

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Introduction

The Grb10 and Grb14 proteins bind to specific phosphorylated sites in the β subunit of tyrosine kinase receptors and regulate the activation of intracellular pathways that promote cellular differentiation and proliferation (1). Studies using human and mouse cells have showed that Grb10 and Grb14 proteins negatively regulate receptor-type tyrosine kinase (2). During oocyte maturation in mammals, some events are regulated by proteins that bind to the receptor-type tyrosine kinase (3). Therefore, the aim of the present study was to evaluate the mRNA expression of Grb10 and Grb14 in bovine cumulus-oocyte complexes (COCs) from follicles at different stages of development.

Materials and Methods

The expression of Grb10 and Grb14 was assessed in COCs from follicles at different stages of development (1-3, 4-5, 6-8 and >8 mm) obtained from ovaries of slaughtered cows. There were three independent replicates with 35 to 40 oocytes per group. Total RNA was extracted, quantified by NanoDrop 1000 spectrophotometer and reverse-transcribed using the Omniscript RTase Kit. The expression of Grb10 and Grb14 was evaluated by real-time PCR using bovine specific primers and normalized to the expression of the housekeeping gene GAPDH. The effect of oocyte development stage on Grb10 and Grb14 gene expression was tested by GLM procedure and LSMeans multi-comparison test.

Results and Discussion

The expression of Grb10 in COCs obtained from 1-3 and 4-5mm follicles was higher than those derived from follicles with 6-8 and >8mm in diameter ($P < 0.05$; Fig. 1.A). Similarly, Grb14 expression was higher in COCs from small-size follicles (1-3mm diameter) and showed a size-related decrease in larger follicles (4-5, 6-8 and >8mm diameter; $P < 0.05$; Fig. 1.B). The present preliminary results shows a negative correlation between the expression profile of both genes (Grb10 and Grb14) with the follicular size, indicating a potential role of these genes in bovine oocyte capacitation during follicular development.

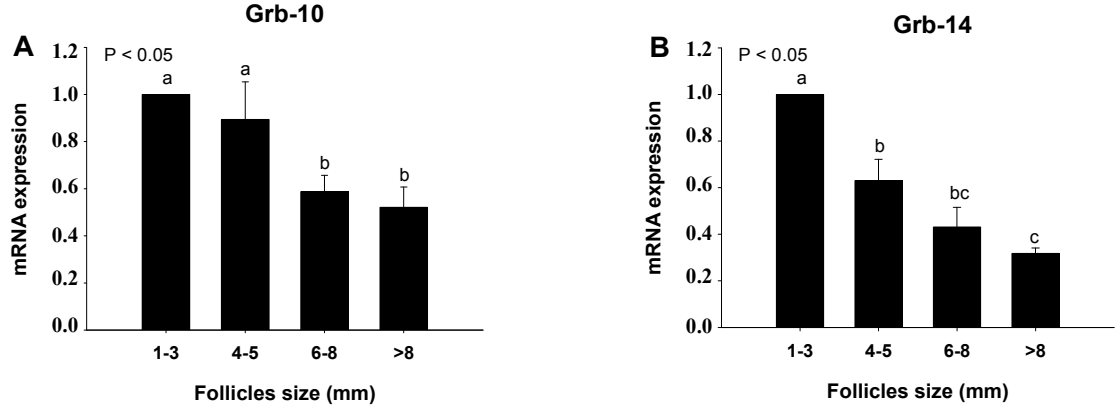

Figure 1. Expression of Grb10 and Grb14 mRNA relatively to the housekeeping gene GAPDH in bovine COCs from follicles at different stages of development. Different letters represent significant differences between follicle sizes ($P < 0.05$).

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Cat sterilization using a zinc-based solution by intratesticular administration: preliminary results

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Introduction

The domestic cat may be the world's most numerous pet, and chemical sterilization could be an alternative to interrupt male reproduction and control over population. A zinc-based solution that is pH neutralized in BioRelease Technologies physiological vehicle (B.E.T. Labs, Lexington, Kentucky, USA) was developed as a chemical sterilizing for intratesticular (i.t.) administration in dogs (1). However, there is little information related to testis structure and function in domestic cats (2) and almost no information regarding chemical sterilization in the domestic cat.

Material and Methods

Eight adult male cats were given 0.2 to 0.3 mL (i.t.) of a zinc-based solution and four control male cats were given, similarly, an (i.t.) administration of 0.2 to 0.3 mL of a saline solution. The testicular width was measured to determinate the dosage of the drug to be given. Before the treatment a semen sample was collected by electroejaculation and spermatozoa concentration was evaluated by light microscopy. Urine was collect following ejaculation, by bladder catheterism, and observed by light microscopy for the presence of spermatozoa. Semen and urine have been collected monthly using the same procedure after the zinc solution administration.

Results and Discussion

In all urine samples, living spermatozoa have been found, what confirms the retrograde ejaculation into the urinary bladder, reported in the literature. Reports show that 15 to 90% of the cat ejaculate can be retrograde into the urinary bladder. Collection of urine by cystocentesis following ejaculation and evaluation of the spermatozoa presence is a useful procedure to confirm a cat sperm output (3). The preliminary results of semen concentration, of the eight treated cats, in the month following the treatment was lower than the previous collection, and no change was seen in the spermatozoa concentration in the control group. The preliminary results of testicular width were smaller in the treated cats. To our knowledge, this is the first report of spermatozoa concentration decrease after treatment with a zinc-based solution in male cats.

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Effect of vitamins A and E administration on oocyte production and early development of bovine embryos

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Introduction

Several different factors can affect the success of an *in vitro* embryo production (IVP) system. We evaluated the effect of a single intra-muscular injection of vitamins A and E on oocyte quality and yield and early embryo development in *Bos indicus* cows.

Materials and Methods

Twenty two *Bos indicus* cows weighing between 450 and 600 kg were subjected to ovum pick-up (OPU). Oocytes were collected in Dulbecco's PBS (DBPS) containing heparin and antibiotics, counted and classified (1). Viable oocytes were taken to the lab, *in vitro* matured for 24 hours and *in vitro* fertilized using 25 million sperm/mL. After 168 hours (39°C, 5% CO_2), viable embryos were also counted and classified. Then, the cows were treated with a single injection of vitamin A (1,000,000 UI; i.m.) and vitamin E (1 g; i.m.). After 12 days, the cows were again subjected to the same procedure described above. Differences in oocyte yield and embryo development were analyzed using paired *t* test.

Results and Discussion

The 40 OPUs from 20 cows yielded 520 oocytes. After vitamin treatment, cows produced more oocytes (n = 303; $p < 0.01$) compared to the previous OPU (n = 217), resulting in 95 more viable oocytes (31%). From those oocytes, 224 embryos were obtained, being 89 before vitamin injection and 135 after treatment ($p < 0.02$), 36 more embryos (40%) produced. A single parenteral injection of vitamins A and E had a significant positive effect on both oocyte yield after ovum pick and *in vitro* embryo development on *Bos indicus* cows. This effect is probably due to the positive influence of retinoids on oocyte and embryo development (2).

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Expression of BMP15 receptors in bovine granulosa cells of dominant and subordinate follicles

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Introduction

The participation of bone morphogenetic protein 15 (BMP15) receptors in the control of follicular deviation in cattle is not clear. Some studies demonstrated that this protein and its receptors have important roles in the regulation of ovulation rate (1, 2). However, the expression pattern of specific BMP15 receptors in bovine dominant and subordinate follicles during follicle growth has not been shown. The aim of this study was to characterize BMPRII and BMPRIb expression patterns in bovine granulosa cells of antral follicles near deviation.

Material and Methods

Beef cows were synchronized and after estrus detection, follicular waves were monitored daily by ultrasound. Day one of the follicular wave was considered when the largest follicle reached 5 mm. Cows were then ovariectomized on days 2 (n = 3), 3 (n = 4) and 4 (n = 4) of the follicular wave, follicles were dissected and granulosa cells submitted to total RNA extraction. BMPR mRNA expression was measured by real time RT-PCR and normalized to GAPDH. Effect of follicle status (dominant *vs*. subordinate) on BMPRII and BMPRIb mRNA abundance was assessed by paired Student's T test using cow as subject.

Results and Discussion

On day 2, there was no difference between the largest and second largest follicle for both receptors (Fig. 1A and 1B). On day 3, when dominant and subordinate follicles were identified, BMPRIb expression was significantly higher in subordinate follicles, whilst BMPRII expression tended to be higher in the same follicle type. After deviation (day 4), expression of both BMPRII and BMPRIb was significantly higher in the subordinate follicle. These results are in agreement with a previous study reporting high expression of these receptors in atretic granulosa cells in the rat (3). Our findings suggest the participation of BMP15 signaling during follicle selection as previously indicated by the effects of active immunization against BMP15 in cows (1). As reported in the ewe, it seems that a reduction in BMP15 signaling occurs during final differentiation of bovine ovulatory follicles (2). In conclusion, BMP receptors are regulated during antral follicular development and their expression is increased in subordinate follicles around the expected time of deviation and beginning of atresia.

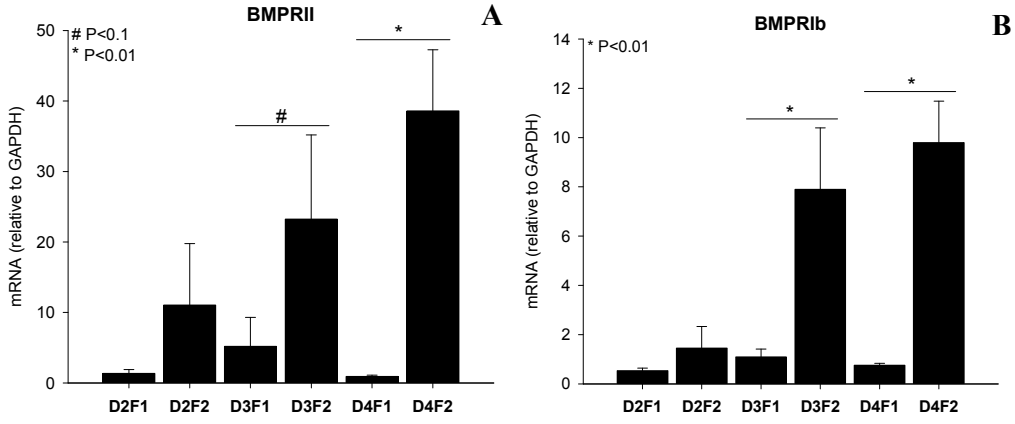

Figure 1. Expression of BMPRII (A) and BMPRIb (B) in dominant (F1) and subordinate (F2) follicles during follicular development.

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Effects of different temperatures on Leydig cells and spermatogenesis

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Introduction

In mammals, despite the temperature being considered a crucial modulator of reproductive activity, there are many unsolved questions regarding the effect of this important factor on the morphofunctional aspects of the testis in this vertebrate class (1). Studies recently developed in our laboratory with fish (tilapias; *Oreochromis niloticus*) have shown that different temperatures are able to influence the testis somatic and germinative components, such as their mitotic/apoptotic activity and the duration of spermatogenesis (2). In this context, the main objective of this study was to evaluate the effects of different environmental temperatures (16, 23 and 32°C) on the structural components of the testis, germ cell apoptosis, duration of spermatogenesis and spermatogenic efficiency in Swiss mice (*Mus musculus*).

Materials and Methods

Eighteen sexually mature Swiss mice were utilized. The animals were exposed to a photoperiod of 12L:12D and allocated in three different experimental groups that were kept in the vivarium at 16°C, 23°C (control group) and 32°C, during three weeks. In order to estimate the duration of spermatogenesis, three animals from each group received intraperitoneal injections of tritiated thymidine (1μCi/g/BW). Testes were perfused-fixed with 4% buffered glutaraldehyde and were routinely processed and embedded in plastic for autoradiographic, histological and stereological analyses.

Results and Discussion

The data found demonstrated that, at 32°C, the Leydig cell volume density (LCVD) and nuclear volume (LCNV) were significantly lower ($p < 0.05$) than the control group. Higher number of apoptosis ($p < 0.05$) in spermatocytes also occurred at this temperature during meiotic divisions, resulting in lower ($p < 0.05$) meiotic index (round spermatids per each primary spermatocytes), Sertoli cell efficiency (spermatids per each Sertoli cell), and spermatogenic efficiency (daily sperm produced per gram of testis). In order to better understand these alterations, we are currently developing studies aiming to evaluate the relationship of Leydig cells activity and germ cells apoptosis. In addition, at 32°C we observed faster ($p < 0.05$) duration of spermatogenesis (~15%) and higher frequency of the pre-meiotic and meiotic phase ($p < 0.05$) of spermatogenesis, suggesting that the heat stress can affect germ cells, mainly during the meiotic divisions. Regarding the group maintained at 16°C, in comparison to the control group we observed fewer significant alterations in spermatogenesis. In this way, the main findings were that the LCVD and the LCNV were significantly higher ($p < 0.05$) and lower ($p < 0.05$) germ cell apoptosis was found during meiosis. The duration of spermatogenesis in this group tended to be slower ($p > 0.05$) and the frequencies of the stages of seminiferous epithelium cycle were more concentrated ($p < 0.05$) in the post-meiotic phase of spermatogenesis. However, the spermatogenic efficiency in this group was not different from that obtained in the control group. Taken together, in accordance with the literature our data show that the temperature is an important factor for spermatogenesis homeostasis, more specifically germ cell loss and sperm production. However, in the present study we demonstrated that this important environmental factor is also able to alter the stages frequencies, duration of spermatogenesis, and Leydig cells function, particularly higher temperatures.

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Estrogenization of gerbil prostate during development is a predisposing factor to lesions in male and female adult

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Introduction

There is an increasing number of researches suggesting that the embryonic phase is likely a critical period of prostatic development, and that the hormonal imbalances are the main cause of alterations in this gland throughout life (1, 2, 3). Moreover recent studies have shown that exposure to synthetic estrogen during intrauterine life is the main factor for appearance of prostatic diseases in the adult and senile life (4, 5). This study aimed to analyze histopathological and stereological aspects of prostate of female and male adult gerbils exposed to estrogenic agent (17α-ethinylestradiol- EE) during the gestational period.

Material and Methods

Five pregnant females received only oil vehicle (control group) and five pregnant females received ethinylestradiol (EE group) daily (10 μg/Kg) in the 17th until 19th day of pregnancy, which is the period when prostatic morphogenesis initiates (6). After the intrauterine estrogenization, the male and female offspring were separated. Each of these groups contained five males and five females. When animals completed 120 days of age, they were sacrificed and the prostate as well as blood samples were collected. Prostatic fragments were processed and stained by hematoxylin-eosin method for stereological (relative volume of epithelium, lumen, muscular stromal and non-muscular stromal compartment) and histopathological (incidence and multiplicity of lesions: hyperplasia, metaplasia and prostatic intraepithelial neoplasia - PIN). Statistical sigificance was considered at the $p \leq 0.05$ level.

Results and Discussion

Serological analysis revealed an increase in estradiol levels of adult males and females exposed to EE during embryofetal period. These data suggest that altered estrogen levels can be the main cause for the appearance of prostatic alterations verified in adult animals. Between these alterations it was observed a decrease of the luminal compartment of prostate and an increase of muscular compartment in the EE group of male. Moreover, non-muscular stromal compartment was reduced in the EE group of females when compared with the control group. Furthermore a high incidence of metaplasia (50%) in the EE group of females was observed. This study suggested that these alterations were mediated by action of alpha estrogen receptor (ERα) in the prostatic epithelium and stroma (7). In both males and females exposed to ethinylestradiol was possible to observe a high presence of PIN (males: 100% and females: 75%). Thus, we propose that ethinylestradiol#exposure during the intrauterine life may be a predisposing factor for development of prostatic diseases in male and female adult life. However, more studies should be accomplished to elucidate the real action of this endocrine disruptor on the prostatic development of both sexes.

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The expression of Grb10 and Grb14 during antral follicle development in cattle

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Introduction

There are several growth factors involved in the regulation of the reproductive functions including follicular development. Among them are the proteins that bind to the receptor-type tyrosine kinase such as IGF-I (Insulin-like Growth Factor I) and insulin (1). Previous studies have shown that the proteins Grb10 and Grb14 act by negatively regulating receptor-type tyrosine kinase (2). However, the role of these proteins in the regulation of ovarian physiology was not yet investigated. Therefore, this study was conducted to assess the expression and regulation of Grb10 and Grb14 during antral follicle development in cattle.

Materials and Methods

In the first experiment, the expression of Grb10 and Grb14 was assessed in granulosa and theca cells collected from follicles at different stages of development (3-5 mm, 6-8 mm and >8 mm) using ovaries of slaughtered cows. In the second experiment, Grb10 and Grb14 expression was assessed in granulosa cells of dominant and subordinate follicles obtained in vivo from cows on Days 2, 3 and 4 of the follicular wave. Day 0 was defined as the last day when the dominant follicle was less than 5 mm in diameter, as described by Evans & Fortune (3).

Results and Discussion

Proteins Grb10 and Grb14 were expressed in both granulosa and theca cells of bovine follicles at different stages of development (data not shown). In the second experiment, there was lower expression of Grb10 at day 4 and Grb14 at days 3 and 4 of the follicular wave in dominant compared to subordinate follicles ($P < 0.05$; Figure 1). Furthermore, it was observed a negative correlation between the expression of Grb10 and Grb14 with the expression of aromatase ($P < 0.05$). Thus, this is to our knowledge the first study to characterize the expression and regulation of Grb10 and Grb14 in granulosa and theca cells during follicular wave in cattle. These findings suggest that Grb10 and Grb14 expression is regulated during antral follicle development and, therefore, potentially involved in the control of follicular wave in cattle.

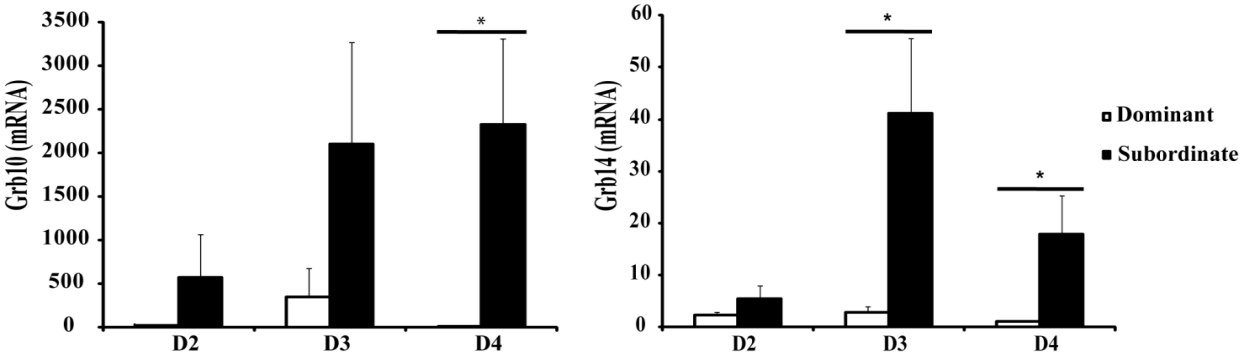

Figure 1. Expression of Grb10 and Grb14 in granulosa cells of follicles obtained on day 2 (D2), three (D3) and four (D4) of the follicular wave in cattle. Open bars represent dominant follicles and black bars represent subordinate follicles. Asterisk (*) indicates statistical difference between largest and second largest follicle within the same day ($P < 0.05$).

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Financial Support: CAPES and CNPq.

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Concentration of tbars in holstein cattle follicular fluid during the summer and the winter

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This study was conducted on two dairy farms during the summer and winter of 2009. Heifers, peak lactation and repeat-breeder Holstein cows were submitted to a protocol to synchronize follicular wave emergence. On a random day of the estrous cycle defined as Day 0, all animals received a norgestomet ear implant (Crestar®, Intervet, Brazil), 2 mg estradiol benzoate (Sincrodiol®, OuroFino, Brazil), 50mg P4 (OuroFino, Brazil) and 150µg D-cloprostenol (Sincrocio®, OuroFino, Brazil) i.m.. The implant was removed on Day 5 followed by ovum pick up. For each animal, the conical tube containing follicular aspirate was transported to the laboratory and the cumulus–oocyte complexes were washed using a 75 µm filter (Watanabe Tecnologia Aplicada, Cravinhos, SP, Brazil) and 10 ml of DPBS supplemented with 1% (v/v) FCS. The oocytes were submitted to in vitro embryo production and the follicular fluid was collected and kept frozen until assayed to quantify the lipid peroxidation marker malondialdehyde, with the thiobarbituric acid reactive substances (TBARS) method. Statistical analyses were performed with PROC GLIMMIX of SAS. The blastocyst production was reduced during the summer for all categories (Fig.1). Heifers, peak lactation and repeat-breeder cows had similar concentration of TBARS (average 109.17 ± 4.87 ng/mL). However different TBARS concentrations were observed during summer and winter (Fig.2).

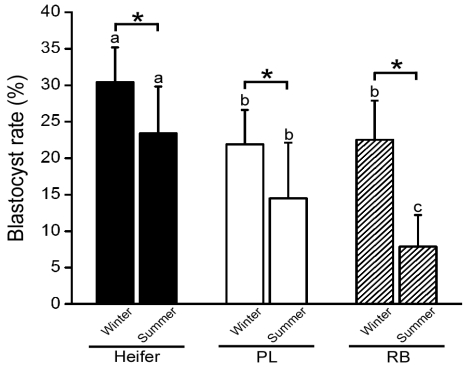

Figure 1. Blastocyst rate seven days post in vitro insemination of Holstein cattle of different categories during summer and winter. Interaction season-category ($P < 0.0001$); mean (±S.E.) values within season ($a \neq b$) and within category (*) differ significantly ($P < 0.0001$).

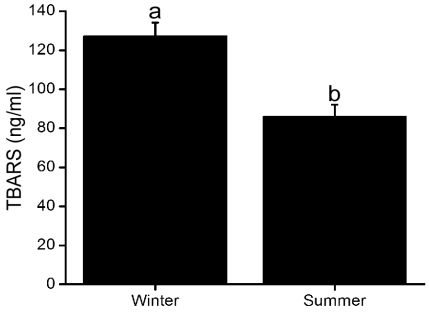

Figure 2. Concentration of TBARS (ng/mL) in the follicular fluid of high-producing Holstein cattle submitted to ovum pick up during the summer and the winter ($a \neq b$; $P < 0.0001$).

It has been reported that embryo production and pregnancy rates are reduced during the summer heat stress. Although high reactive oxygen species (ROS) levels may damage oocytes and embryos, moderate levels have been associated to a beneficial effect on early development. Hence, ROS concentration in follicular fluid has been suggested as a gestational marker in humans. Thus, the findings of the present study are in agreement with this beneficial role of ROS on early development, suggesting that the lower levels of ROS during summer heat stress may be involved in damaging oocyte quality in high-producing Holstein cattle.

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Uterine inflammatory response after artificial insemination with different quality semen evaluated by combinations of fluorescent probes in equines

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Introduction

The fertility is one of the most important characteristic for the productivity of equine production. After deposition of semen in the female reproductive tract an inflammatory response occurs, physiological and transient, represented by the uterine contractility, influx of neutrophils and increase of the blood perfusion (3). The endometrial cytology and ultrasonography with color Doppler mode are validated methods to assess this influx of neutrophils (1) and uterine vascular perfusion (2), respectively. Thus, the objective of the present study was to compare the inflammatory response between the deposition of low quality semen (higher number of damaged sperm) and of good quality semen. Authors expected a greater inflammatory response associated with a low quality semen.

Materials and Methods

Twelve mares without endometritis predisposition were selected previously, considering the reproductive history and gynecological exam, and divided into three groups: Group inseminated with high quality semen (40-60% of intact sperm, n = 4), group inseminated with low quality semen (10-30% of intact sperm, n = 4) and control group (not inseminated, n = 4). Follicular development was monitored daily, and in the presence of a follicle ≥35 mm chorionic gonadotropin (hCG) was administrated to induce ovulation. The evaluation of uterine environment was held with color Doppler mode transrectal ultrassonography and with gynecologic brush uterine cytology in three different periods: before the ovulation (soon after de hCG administration, 30 hours before the A.I.), 6 h after de A.I. and 24 h after the A.I. Pregnancy diagnosis was performed 14 days after A.I. The variables were submitted to analysis of variance and LSD test at 5% level of significance and the fertility data was analyzed with X^2.

Results and Discussion

There was no effect of treatment (i.e., quality of semen) nor effect of the interaction of treatment by time for any of the variables analyzed ($p > 0,05$). However, there was an increase in blood perfusion and influx of neutrophils across time.. The fertility of the high quality semen was 75%, whereas for the low quality semen was 25%, but no statistical difference was noted between these values, probably due to the low number of experimental animals. This experiment was an initial step towards the use of color Doppler mode ultrasound in mare reproduction studies.

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Evaluation of estrus behavioral and ovulatory dynamics of Toggenburg goats during the reproductive transition season

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Introduction

The development of the goat industry is showing a global cycle of growth, especially in developing countries where the largest herds are located. Under this perspective, there is a crescent need to assist the reproduction of these animals (1). The objective of the study was to evaluate the estrus behavioral and ovulatory dynamics after the use of intravaginal progesterone-relasing implants for three different periods (6, 9 and 12 days) during the transition season in Toggenburg goats.

Material and Methods

The study was conducted in the city of Piau (21°35' S latitude and 43° 15' W longitude), at Zona da Mata, Minas Gerais, Brazil, in January and February (summer). Thirty animals were chosen after undergoing ultrasound assessment, according with body condition (≥2,5) and weight (≥28kg) and lactating or non- lactating status. The treatment groups were formed according to the time of permanence of the intravaginal implant (CIDR-G®, Pfizer-Animal Health, São Paulo, Brazil), 6 (n = 10), 9 (n = 10) to 12 (n = 10) days. At the time of implant insertion all animals received 5 mg dinoprost (Lutalyse®, Pfizer - Animal Health, São Paulo, Brazil) paravulvar and 24 hours before the implant removal, 200 IU eCG (Novormon®, Schering-Plough Animal Health, São Paulo, Brazil) i.m. Estrus detection and natural mating began 12 h after implant removal and ovulations were monitored by ultrasonography (Aloka SSD 500®, Tokyo, Japan) every eight hours. Ovulations were determined by the presence of follicles with an irregular shape and decreased diameter. Statistical analysis was performed using the Kruskal - Wallis (onset of estrus, duration of estrus, interval from implant removal to ovulation and interval from estrus to ovulation) or x^2 test (pregnancy rate) using the STATA program at 5% probability.

Results and Discussion

No effect of duration of implant use was detected for the following variables: onset of estrus, duration of estrus, interval from implant removal to ovulation, interval from estrus to ovulation and pregnancy rate. The results are presented on Table 1.

Table 1. Reproductive parameters evaluated in Toggenburg goats after hormone treatment with progesterone implant during 6, 9 and 12 days (Mean ± SD).

Evaluated reproductive parameters	Treatments		
	6 days	9 days	12 days
Onset of estrus (h)	24.00 ± 3.46	25.67 ± 5.29	25.18 ± 4.85
Duration of estrus (h)	42.92 ± 25.67	32.00 ± 14.70	31.63 ± 22.30
Interval from implant removal to ovulation (h)	53.33 ± 21.96	43.67 ± 10.09	45.54 ± 12.04
Interval from estrus to ovulation (h)	29.33 ± 22.39	18.00 ± 9.58	20.36 ± 12.13
Pregnancy rate (%)	50.0 (5/10)	50.0 (5/10)	50.0 (5/10)

According to our findings, all three treatments, regardless of exposure time (6, 9 and 12 days) to progesterone, are efficient to induce and synchronize estrus in Toggenburg goats during the transition season. Therefore, the six-day treatment should be preferred because it is shorter and more appropriated to be used in the field practice.

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Reproductive isolation as the key for chromosome speciation in Red Brocket Deer (*Mazama americana*)

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Introduction

Red brocket deer (*Mazama americana*) has a wide distribution in Brazil and a complex evolution due to a chromosome polymorphism, which poses many questions about taxon validation. This species is genetically characterized by distinctive karyotypes with some geographic coherence. Molecular and cytogenetic evidence (1) suggests the existence of a cryptic species complex. The aim of this work was to analyze the fertility of pure and hybrid males, by histological and semen evaluation.

Materials and Methods

The crossing of four cytotypes produced seven bucks. The controls do not have difference between parental autosomes (dpa): 2n = 45 x 2n = 44 (animal 1) and 2n = 53 x 2n = 52 (animal 2). The others hybrid males have three with dpa=2, animal 3 and 4 (2n = 45 x 2n = 42), animal 5 (2N = 51 x 2N = 52); the animal 6 with dpa = 8 (2N = 45 x 2N = 52) and animal 7 with dpa = 11 (2n = 43 x 2n = 52). Animals were anesthesiated (5 mg/kg kethamine and 1 mg/kg xylazin), on 12 and 18 months old, and submitted to electro-ejaculation. Semen was evaluated by traditional parameters: volume (µl), motility (%), vigor (0-5) by optical microscopy; a sample was fixed in saline formol (1:200) and evaluated to sperm concentration using Neubauer Chamber and morphology by wet preparation with a phase contrast microscope, according to CBRA (2) and Blom (3). After semen collection, on the second restrain (18 months old), animals were submitted to a unilateral orchiectomy. Testes were fixed in Bouin´s solution to classical histological procedure, and the presence of different cells was evaluated.

Results and Discussion

Only a few samples of semen was analyzed, which confounded the statistical analysis, but animals 6 and 7 (dpa > 2) were azoospermic at both collections (Table 1). In addition, the testicular histology of these animals presented only early gametic cells (spermatogonia and rarely primary spermatocytes) with many degenerate seminiferous tubules with apoptosis, which suggests that a great difference of parental autosomes results in infertility, as suggested (4). Animals 3, 4 and 5 (dpa = 2) presented all stages of gametogenesis, indicating that the distance between parents do not induce significative defects in spermatogenesis. Our results suggest the existence of an effective reproductive barrier between some cytotypes of *M. americana*, characterizing the main system for speciation based on chromosome differentiation in the *Mazama* genus.

Table 1. Means and standard deviation of physical and morphologic sperm quality in red brocket deer bucks according to difference of parental autosomes (DPA)

DPA (n)	Collection (Months)	Volume (µL)	Motility (%)	Vigor (0 - 5)	Concentration ($x10^6/mm^3$)	Total Defect (%)
DPA = 0 (2)	12	100.0 (0.0)	37.5 (53.0)	2.0 (2.8)	1.2 (1.7)	7.5 (10.6)
	18	135.0 (35.4)	77.5 (3.5)	3.5 (0.7)	3.8 (0.3)	20.5 (0.0)
DPA = 2 (3)	12	33.3 (57.7)	40.0 (36.1)	2.2 (1.8)	0.9 (0.6)	54.5 (47.5)
	18	246.7 (64.3)	68.3 (12.6)	2.7 (0.6)	3.5 (2.5)	30.0 (26.1)
DPA > 2 (2)	12	50.0 (70.7)	-	-	-	-
	18	100.0 (141.4)	-	-	-	-

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Characterization of serum levels of 17β-estradiol and progesterone in human induced labor with dinoprostone during late gestation

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Introduction

The maintenance of human pregnancy requires a hormonal coordination during gestation and labor. Recent research has shown that potential key factors involved in the process of human parturition are the changes on progesterone and estrogen levels and increased production of prostaglandins and oxytocin (1). Clinically, the administration of PGE2 analogues is used to induce labor with different individual responses (2). However, determinant factors to non-responsiveness to induction of the labor are not well understood in pregnant women. The objective of this study was to determine the relationship of the maternal serum levels of 17β-estradiol (E2) and progesterone (P4) in pregnant women at term treated with dinoprostone (PGE2 analogue).

Materials and Methods

Blood samples were collected from 16 pregnant women at admission for term labor and immediately before delivery. All women agreed to participate of the research according to Research Ethic Committee of UFSM. The treated group (TG) was divided in patients that responded to induction using an intravaginal device with 10mg dinoprostone (positive TG; n = 11) and patients that did not respond to the induction (negative TG; n = 5). As a control, blood samples were collected from pregnant women at term that had labor spontaneously (n = 13) and women that had no labor spontaneously and were submitted to c-section (n = 5). The total blood was centrifuged and plasma serum was used to measure E2 and P4 concentrations using a chemiluminescence enzyme-linked immunosorbent assay (CELISA).

Results and Discussion

Women treated with dinoprostone had serum levels of the E2 reduced after induced-labor (Fig. 1A) but we observed that P4 serum levels were also affected in response to dinoprostone. When each group was compared, levels of P4 were reduced significantly only in patients responsive to labor induction (Fig. 1B). E2 levels were reduced in both treated groups that responded and did not respond to induction with dinoprostone (Fig. 1C). In conclusion, treatment with prostaglandin analogs decreases serum E2 irrespective of labor induction. Moreover, P4 levels are reduced only in patients that are responsive to dinoprostone induced-labor and this could be related to the mechanism of labor-induced failure.

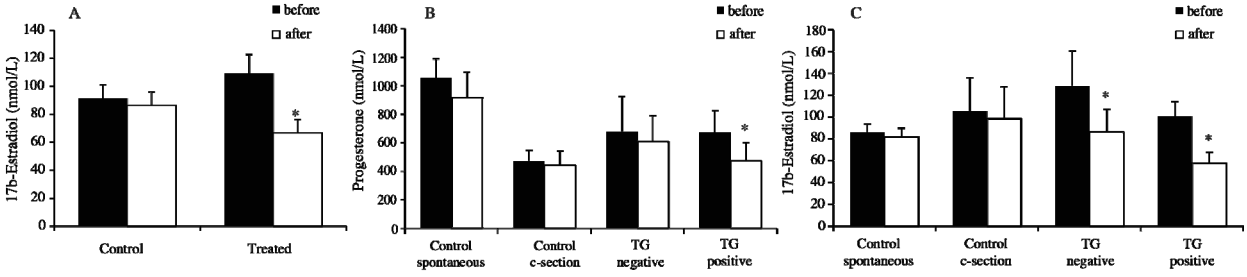

Figure 1. Relative maternal serum levels (mean ± s.e.m.) of the (A) 17β-estradiol in pregnant women at term without induction with dinoprostone (control) and after induced labor with dinoprostone (treated) before and after labor; (B) Progesterone and (C) 17β-estradiol serum levels in pregnant women at term that responded to dinoprostone induction (TG positive) and patients that did not respond to the induction (TG negative) compared to spontaneous labor (Control spontaneous) or not in labor and submitted to c-section (Control c-section). (*) Asterisk indicates statistical difference (before vs. after; $P < 0.05$) by paired Student's T-test using women as subject.

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COX-2 activates MAPKs which are responsible for the lifespam of corpus luteum in bovine

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Introduction

Corpus luteum (CL) is a transient gland responsible for progesterone production, formed from the ovulatory follicle (1) and its main luteolytic agent is prostaglandin F2α (PGF2α; 2). Prostaglandin F receptors (FP) for prostaglandin activate the MAPKs, enzymes that phosphorylate specific serine-threonine sites, including ERK 1/2 (3). These kinases have the ability to decrease progesterone synthesis, blood flow in the CL (4) and cell survival factors like Bcl-2 (5). The aim of the present study was to evaluate the relationship between COX-2 activity, MEK/ERK 1/2 and Bcl-2 during formation of CL and luteolysis.

Materials and Methods

Ovaries were collected from a slaughterhouse of Goiás (Brazil) and classified into seven groups: 1-3, 4-6, 7-9, 10-12, 13-15, 16-18 and 19-21, according to the estrous cycle phases (6). The ovaries were fixed in Bouin's solution for histological processing. Paraffin blocks were cut 5 mm thick and subjected to immunohistochemistry with specific antibodies that recognize the active (phosphorylated) forms of MEK 1/2 and ERK 1/2, or the proteins COX-2 and Bcl-2. Counting was performed in ten fields in each slide, assessing antibody labeling with a light microscope and determining grading scores as 1 (low), 2 (moderate) and 3 (intense).

Results and Discussion

The ovulation day was considered as day 0. Thus, group 1-3 were experiencing the formation of the corpus luteum. At this point, samples with intense score for COX-2 were 100%, probably related to high PGF2α concentration in ovulatory follicular fluid until 24 hours after ovulation (7). At this time, MEK 1/2 presented low scores, due to its high levels in preovulatory follicles. The activation of ERK by MEK required 24 hours (8), so the activity of ERK increased in group 4-6. This same group showed a 24% increase in the number of samples with moderate and intense score for the protein Bcl-2, when compared to group 1-3, probably being modulated by ERK. In the group 7-9, it was observed a 18% increase in the number of samples with scores moderate to intense for COX-2, and this is the moment when the CL reaches maximum production of progesterone (9). In this group, as well, there was an increase of 55% of samples with moderate and severe scores for MEK and 66% in the subsequent group, 10-12, to ERK. In group 13-15 there was a 50% increase in the number of samples that showed intense scores for COX-2. This point is considered the luteolytic signal (10). At this time, there was an increase in MEK activity of 30%, which activates a mechanism of downregulation for progesterone synthesis by inhibiting important steroidogenic enzymes (11) and starts the functional luteolysis. In group 16-18, 50% of samples had moderate scores for ERK, whereas there was a 24% decrease in Bcl-2 activity. It was assumed that in the period 13-15, there is a signal for functional and structural luteolysis, via increase of active forms of MEK/ERK 1/2, which also influence the decrease in anti-apoptotic factor Bcl-2. It was concluded that there was a change in expression and/or activation of these enzymes, showing an association between the profiles of COX-2, MEK/ERK 1/2 and Bcl-2 during development, survival and death of the corpus luteum.

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Production of *GDF9* and *BMP15* bovine hormones in homologue culture system

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Introduction

Recently, many studies are contributing to improve the knowledge about paracrine factors actions inside the follicle, especially regarding *GDF9* and *BMP15* that induce the somatic cells growth and differentiation, as well as the follicle development (1). Both, *GDF9* and *BMP15*, are expressed by the oocytes in all follicular developmental stages in mammals (2) and are fundamental to activate the primordial follicles and, subsequently, to participate in all stages of follicular development (3). However, the influence of endocrine and paracrine signaling over somatic cells growth and differentiation are not completely understood (4). All researches in this area use hormones and growth factors in heterologous culture systems, which have produced largely divergent results (5, 6). In order to minimize the heterologous effect, this study was carried out to express bovine *GDF9* and *BMP15* genes in homologous culture systems to better understand the paracrine signaling between oocytes and granulosa cells (GC).

Material and Methods

To express *GDF9* and *BMP15* in bovine cells, total RNA was extracted from bovine oocytes with Trizol reagent, following by RT-PCR of the genes ORF (open reading frame) with specific primers. The fragments were purified, cloned in pGEM-T easy vector and sequenced. After sequence confirmation, the inserts were excised from pGEM-T easy with restriction enzymes and cloned into mammal expression vector pCINeo. Then, the vector containing the fragment was transfected by lipofection (Lipofectamine 2000-Invitrogen) to MDBK bovine cells, and selected with G418 antibiotic for vector integration. After selection, DNA was extracted with salting-out protocol and the RNA was extracted with Trizol to confirm the vector insertion and the homologous gene expression. Subsequently, western blotting was used to confirm the *GDF9* and *BMP15* proteins expression.

Results and Discussion

The PCR of genomic DNA, as well as the cDNA and western blotting of transgenic MDBK cells showed that *BMP15* and *GDF9* bovine proteins were correctly integrated in the genome and expressed in the homologous bovine cell culture system. This is the first work showing the expression of recombinant *GDF9* and *BMP15* bovine genes in bovine cells. Research using medium conditioned with *GDF9* and *BMP15* have shown their involvement in the development of preantral follicles (7), modulation of activin A activity (8), and increasing bovine IVF embryos rates (5). However, many aspects regarding the influence of *GDF9* and *BMP15* still need further understanding. The species-specific expression system shown here is a valuable tool for the better understanding of paracrine actions of *GDF9* and *BMP15* (9).

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Brain pathways that mediate the 'female effect' in sheep

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Introduction

The 'male effect' in small ruminants, in which the introduction of males induces fertile cycles in seasonally anovulatory females, was first reported by Underwood and colleagues in 1944 (1). This topic has been studied in detail over the last 60 years and reviewed many times, including the recent article by Delgadillo et al, 2009 (2). There is an analogous 'female effect', first described by Lee Sanford's team in Canada in the 1970s (3), in which the introduction of females stimulates the male reproductive system, eliciting a sudden activation of the neuroendocrine axis, evidenced by an increase in LH pulse frequency. The extra LH pulses evoked by the female stimulus immediately enhance the secretion of testosterone, presumably activating the sudoriferous glands, the site of production of the pheromones that are responsible for the 'male effect'. Thus, it appears that the two sexes stimulate each and this enhances the overall outcome with respect to ovulation. We know little about the socio-sexual signals that mediate the 'female effect' or how they exert their actions – indeed, we are not even sure whether they are olfactory/pheromonal, visual or auditory. One way forward is to identify the neural pathways that mediate the responses. We have used the immediate early gene, c-fos, to identify brain cells in adult Merino rams that are activated by the introduction of ewes.

Materials and Methods

During the breeding season, treated rams (n = 4) received fence-line contact with oestrous ewes during a frequent blood-sampling regime, whereas control rams remained isolated. Blood plasma was sampled every 15 min for 4 h before and for 2 h after the time of ewe exposure. After the last sample, the rams were killed with an overdose of barbiturate and brain was removed. Sections of the hypothalamus and preoptic area were processed for Fos immunohistochemistry. All plasma samples were assayed for LH and the data were analysed for pulses.

Results and Discussion

There was no change in LH pulse frequency in control rams, but ewe-exposed rams showed a 3-4-fold response ($P < 0.01$) to the female stimulus (Table). Ewe-exposed rams had 13-17-fold more Fos-immunoreactive cells in the ventromedial hypothalamus ($P < 0.05$) and arcuate nucleus ($P < 0.01$) than Control rams. The medial preoptic area was far more variable and no significant difference was detected.

Treatment	LH pulses/h		cFos immunoreactive cells/mm^2		
	Before	After	Arcuate nucleus	Ventromedial h	Medial preoptic area
Ewe-exposed	0.19 ± 0.12	0.75 ± 0.14	1.87 ± 0.45	0.86 ± 0.28	0.87 ± 0.41
Control	0.25 ± 0.10	0.25 ± 0.10	0.14 ± 0.04	0.05 ± 0.02	0.50 ± 0.40

We conclude that the sites activated in the male hypothalamus by female socio-sexual stimuli are similar to those activated in the female by the male (4). These sites are linked to the output of gonadotrophin-releasing hormone, and thus LH, but the nature of the activated cells is not yet defined.

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The presence of splice variants of prostaglandin transporter protein (PGT) in equine endometrium

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Introduction

Prostaglandins $F_{2\alpha}$ and E_2 play important roles in the reproductive tract of mares. While endometrial $PGF_{2\alpha}$ is essential in cyclic mare on 14 days of estrous cycle to return to estrous (1), the embryonic and endometrial PGE_2 is important in early gestation to recognition of pregnancy (2). These hormones depend of a specific transporter expressed both in producing and target cells, known as PGT (3). This protein has not been identified in equine tissues yet. Therefore, the aim of this work was to characterize PGT mRNA expression in equine cyclic and pregnant endometrium.

Materials and Methods

Endometrium samples in different reproductive phases were collected by biopsy as follows: estrous (n = 6; EE), seven (n = 6; E7) and fourteen (n = 6; E14) days after ovulation and on 14 days of pregnancy (n = 4; Egest). Total RNA was extracted and one step transcriptase reverse real-time PCR was performed in triplicate with primers designed for exons 4 and 5 of equine PGT. After, the dissociation protocol was performed. The levels of PGT expression were normalized by the housekeeping endogenous control 18s. The amplified products were electrophoresed on 1.5% agarose gel, stained with EtBr and quantified with ImageJ (Wright Cell Imaging Facility, Toronto). Computerized analysis for splice sites was performed with Genesplicer (Center of Bioinformatics & Computational Biology, University of Maryland). The data (means ± stander of error mean) were submitted to ANOVA and the averages were separated for the LSD test ($P < 0.05$).

Results and discussion

PGT was expressed at all endometrial samples, according to Fig. 1. In the dissociation curve, it was observed two different peaks in some of the samples studied which following the gel electrophoresis, two different bands were noted, one being around 100bp (WT) and another one around 400bp (a new splice variant called here as SV1). The SV1 relative presence (SV1/WT) was increased in E14 compared with EE. The incidence of SV1 was different between the groups (EE = 100%; E7 = 100%; E14 = 66%; Egest = 50%). Both possible isoforms of equine PGT will be sequenced. The lowest relative value of mRNA for equine PGT found in endometrium of mares in estrous, compared to diestrous is compatible with the physiological importance of $PGF_{2\alpha}$ in the phases of the estrous cycle of mares (1). The largest value in endometrium of pregnant mares may be due to another PGT function, the transport of PGE_2, whose affinity for PGT is similar to affinity for $PGF_{2\alpha}$ (4).

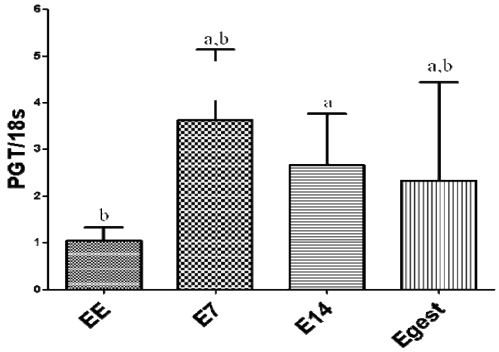

Figure 1. PGT mRNA expression in endometrium of mares in estrous (EE), 7 (E7) and 14 days (D14) after ovulation and in the equine endometrium with 14 days of gestation (Egest). Different letters (a, b, c) indicate significant differences between the endometrial groups ($P < 0.05$).

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Embryo-fetal development in agoutis by ultrasound

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Introduction

Agouti is a small and medium-sized wild rodent, which is distributed in Neotropical forests. The predatory hunting has reduced their existence. It needs studies that help in understanding their biology and conservation (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).

Methods

We studied 27 agoutis undergoing transabdominal ultrasound machine with a Pro Logic 100 and a 7.5 MHz linear transducer. Animals were evaluated throughout gestation (10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 103 days) as to the appearance of the structures of pregnancy.

Results and Discussion

An enlarged uterus was observed at day 10 of gestation at 9.67% and 38.70% in 13 days of gilts. The gestational sac was first observed as an anechoic structure, surrounded by a thin wall and hyperechoic, respectively at 14 and 17 days of gestation, respectively 35.48% and 38.70 in the animals studied, as also related in human (2). The embryo is viewed first as an echogenic structure elongated and eccentrically located within the gestational sac at 20 days of gestation (29.03% of animals). At 22 days this structure was already observed in 64.51% of the agouti rats. At 25 days, an outbreak of eco fluttering inside the embryo can be observed, characterizing the heart rate and fetal viability, seen in 64.51% of the animals. Also at 25 days, a circular structure of heterogeneous architecture and the hypoechoic wall subplacenta shall be observed in 100% of animals. This large structure was firstly described in pregnant rabbits during a morphological study (3). Sprouts limbs and vertebral calcification appear respectively at 27 and 30 days of gestation, viewed as highly reflective structures and forming an acoustic shadow in 48.38% of the animals evaluated. The umbilical cord is a tapered structure, hyperechoic and homogeneous echotexture observed after 30 days of gestation (54.83% of animals). The fetal growth progresses allowing the recognition of organogenesis by ultrasound. The fetal orientation (head and body) was evident in 48.38% of the animals on day 40 of gestation. Similarly, calcification of the skull starts at 45 days of gestation, being seen in 77.41% of the animals studied. The stomach becomes viewed as an anechoic area cavity at 50 days in 54.83% of the specimens, in agreement to the descriptions in dogs (4). The lung and liver are also visualized at 50 days of pregnancy in 54.83% of the animals studied, the first being more hyperechoic in relation to the second. The spinal canal is being viewed at 55 days (45.16%) involved the body of the vertebrae. The kidneys are visualized at 55 days (41.93%) as a hypoechoic structure with an anechoic central pelvis. Large vessels such as thoracic aorta and vena cava flow are visualized at 70 days of gestation (41, 93%). Finally, the intestine and urinary bladder (anechoic structure) are the latest structures observed in the development of gestational agouti. These were found at 85 days of gestation in 38.70% of gilts. Thus, gestational organogenesis of agoutis might be adequately accessed by using ultrasound, this technique being able to generate benefits of great value in maintaining the reproductive management of agouti.

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Random versus imprinted X-inactivation in human placentas

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Introduction

In mammalian females, the transcriptional activity of the XX chromosomes is a tightly regulated and complex process and abnormalities lead to reproductive malfunction. In mice, the placenta maintains a silent imprinted Xp (paternal), whereas the fetus exhibits a random X inactivation, where either the Xp or the Xm (maternal) chromosome is silenced. In humans, the existence of imprinted X-inactivation remains controversial (1). Preeclampsia (PE) is a hypertensive complication of pregnancy characterized by high blood pressure that occurs after 20 weeks of gestation accompanied by proteinuria (2). PE has an unknown etiology involving maternal, paternal and/or fetal factors (3). In this study, we verified the pattern of X chromosome inactivation (XCI) and the parental origin of the inactive X chromosome in normal and preeclamptic pregnancies.

Material and Methods

We analyzed 58 singleton pregnancies. DNA was extracted from blood samples of 40 normotensive and 18 preeclamptic pregnant women. The DNA from the placenta of female fetuses (17 controls and 10 with PE) was also analyzed. According to the *HUMARA* (*human androgen receptor* gene) assay (4), prior to PCR amplification, genomic DNA was digested with *Hpa*II (a methylation-sensitive enzyme). The resulting PCR products were analyzed on a polyacrylamide gel and on an automated sequencer. Data were analyzed by Chi-square and Fisher's exact tests, and by Generalized Linear Model.

Results and Discussion

There was no correlation between the XCI patterns of PE mothers and their respective placentas. When we considered as skewed inactivation values ≥70% of the predominant allele, statistical analysis of our two groups (control and PE) revealed no significant differences. However, despite the small cohort, when the clinical findings were analyzed considering skewed inactivation values ≥80%, we observed that 2/10 (20%) blood informative cases of women with PE and 3/16 (18.75%) of the control group had skewed XCI. In the placenta, we found skewed XCI in 3/9 (33%) cases of the PE group and 4/15 (26.6%) in the control group. When we considered ≥90%, we found 1/10 (10%) in blood and 2/9 (22.2%) in the placenta of PE. In the control group, we found skewed XCI only in one placenta [0/16 (0%) blood and 1/15 (6.6%) placentas]. The XCI was mostly random, but in the control group we also found skewed inactivation (Xp or Xm). Interesting, for the PE placentas, in all cases of skewed XCI, the predominant inactive X was the Xm. Considering that in other mammals the placental imprinted X is the Xp, the extreme opposite Xm preferential inactivation in humans could have some consequences in placental development. These results show a potential role of the XCI in the PE development and could bring some evidences of the existence of imprinted XCI in humans.

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Phenazine ethosulfate effect in the development and lipid content of *in vitro* produced bovine embryos

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Introduction

The reduced cryotolerance of in vitro produced bovine embryos (IVPE) is frequently associated with lipid accumulation in the cytoplasm (1). Phenazine ethosulfate (PES) is a metabolic regulator that inhibits fat acids synthesis and favors the pentose-phosphate pathway because it oxidizes NADPH to $NADP^+$ (2). The aim of this study was to test the supplementation of PES starting at D2.5 and D4 of the culture medium of IVPE.

Material and Methods

Oocytes were matured and fertilized *in vitro* (D0). The zygotes (*Bos indicus*) were divided in 3 groups: ***In vitro* Control**- SOF+2.5%FCS (n = 1253); **Group PESD2.5** -SOF+2.5%FCS+0.3 µM PES starting at D2.5 (n = 1230); **Group PESD4** -SOF+2.5%FCS+0.3 µM PES starting at D4 (n = 1215). The embryos were cultured for seven days at 38.5°C in 5%O_2, 5%CO_2 and 90%N_2. Embryos (*Bos indicus*) recovered from superstimulated cows were used as ***in vivo* Control** (n = 45). Embryo lipid content was assessed through Sudan black B stain. Data were analyzed by ANOVA followed by LSD using PROC GLIMMIX (SAS, Cary, NC) and are expressed as means ± SEM.

Results and Discussion

The use of PES in the culture medium starting at D4 did not affect ($P > 0.05$) blastocyst production (43.0 ± 2.0^b) and cell number (144.8 ± 4.7^A) compared with the control group (42.0 ± 2.8^b and 136.6 ± 5.5^A), respectively. However, despite presenting a similar number of cells (138.2 ± 6.2^A), PESD2.5 reduced ($P < 0.05$) blastocyst production (35.0 ± 2.3^a). The addition of PES to the media, on both D2.5 and D4 reduced the content of medium and large lipid droplets. Nevertheless, the addition of PES on D2.5 was able to reduce the number of small droplets and to promote an even more pronounced reduction ($P < 0.05$) of large droplets. Despite the decrease in the lipid droplet number achieved by the use of PES, the *in vivo* control group had the lowest ($P < 0.05$) lipid content (Fig. 1).

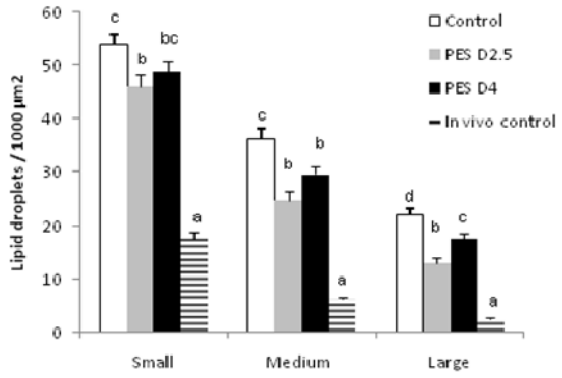

Figura 1. Main effect of treatment groups (Control, PES D2.5 and PES D4) for the number of small (<2 µm), medium (2 - 6 µm) and large (>6 µm) cytoplasmatic lipid droplets in bovine embryos (mean ± SEM); letters on the top of columns (a, b, c, d) refer to differences ($P < 0.05$) between groups in the categories of small, medium and large droplets. N = 60 - Control, PES D2.5 and PES D4 groups. N = 15 – *in vivo* control group.

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Volumetric proportion of placentome structural components of Nellore cow throughout gestation: preliminary results

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Introduction

The placenta is a vital organ for reproduction, responsible for the maternal-fetal metabolic interchange throughout gestation period. The trophoblast and the maternal epithelium form the placentome through morphological changes resulting in final maturation and future placental release (3). The study and comprehension of placental morphology and physiology has great practical importance, to approach placental retention and puerperal problems related to it. The objective of this work was to evaluate placentome structural histomorphometric characteristics throughout the gestation of Nellore cows.

Material and Methods

Placentome fragments from 21 Nellore cows distributed into three fetal developmental stage groups were collected, fixed in formalin and included in paraffin. Tissue samples were stained with Hematoxylin-Eosine and Gomori Trichrome for light microscopy evaluation. The volumetric proportion of placentome structural components are shown in Table 1.

Results and Discussion

Table 1. Volumetric proportion (%) of placentome conjunctive tissue (Conj), caruncular ephitelial cells (CEC), maternal tissue (MT), fetal tissue (FT), binucleate trofoblast cells (CTB) and fetal maternal vessels (V) in the first (0-100 days), second (100-180 days) and third (180-270 days) trimester of pregnancy

Gestation period	Conj.	CEC	MT	FT	CTB	V
0-100	18.52 ± 2.58^{a}	24.4 ± 4.42^{a}	18.71 ± 5.82^{a}	22.48 ± 2.76^{a}	4.02 ± 1.04^{a}	2.45 ± 1.31^{a}
100-180	31.65 ± 7.41^{b}	15.11 ± 4.14^{b}	$8.85 \pm 4.55^{a,b}$	28.82 ± 6.40^{b}	3.08 ± 0.92^{a}	3.71 ± 1.30^{a}
180-270	34.37 ± 3.78^{b}	10.74 ± 3.16^{b}	2.14 ± 1.53^{c}	36.31 ± 7.88^{b}	4.34 ± 1.85^{a}	5.42 ± 1.52^{a}

[ab]Numbers with different letters in the same column differ statistically ($P < 0.05$).

The results above demonstrated differences in the volumetric proportion of maternal cells among gestation trimester, with clear and sharp reduction in the third trimester. These results are in agreement with previous findings (1, 3, 4). The other variables showed statistical differences between the first and second trimester, with no change between the second and third trimesters (4, 5). A gradual increase in the volumetric proportion of vessels was found in the fetal tissue (2). This finding can be explained by the fact that in this present work there was no separated volumetric proportion evaluation between maternal and fetal tissues. It was also verified significant variation between conjunctive and fetal tissues collected in the first and second trimesters. Fetal tissue remained similar between the second and third trimesters, with a gradual increase as the parturition approached. This result is similar to previous findings (2). Caruncular epithelial cells showed significant difference only between the first and second trimester of gestation, although with consistent decrease of these cells throughout the gestation. The preliminary results indicate that there are similarities between *Bos Indicus* and *Bos Taurus* regarding the volumetric proportion of the placentome components, although further studies are needed to see for eventual physiological difference between the racial groups

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New variants in the imprinted *CDKN1C* gene

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Introduction

The hypertensive disorders of pregnancy (HDP) [chronic hypertension (CH), gestational hypertension (GH), pre-eclampsia/eclampsia (PE/E) and PE/E superimposed on chronic hypertension] are globally, among the leading causes of maternal and fetal death (1). Occurring only during pregnancy, the etiology of HDP remains unclear, but there are increasing evidences showing the influences of genetic and epigenetic predisposition in the development of these disorders (2, 3). Several studies are attempting to associate candidate genes with epidemiological data to the transmission model (3). The *CDKN1C* (*cyclin-dependent kinase inhibitor 1C*) human gene, also known as $P57^{Kip2}$, suffers genomic imprinting (maternal allele expressed) and is mapped on chromosome 11p15.5 (4). Along in the same mapped region, are localized the *IGF2* (*insulin-like growth factor-2*), *H19*, *KvLQT1* e *LIT1* imprinted genes (5). The *CDKN1C* plays an important role in inhibiting trophoblast proliferation (6). Mouse models have shown that variances in PE may be induced by proliferation of the trophoblast resulting from decreased expression of this gene and causing abnormal placentation (6).

Material and Methods

This study examined the association of HDP with variants of *CDKN1C* gene. Three hundred and sixty eight pregnant women (168 in the control group, 66 with GH, 43 with CH, 75 PE and 4 E and 11 with PE/E superimposed on CH). We also analyzed 302 healthy subjects (204 females and 98 males aged between 18 and 35) of the general population as a second control group. After DNA extraction we genetic screened the groups using molecular biology techniques (PCR - polymerase chain reaction, SSCP - Single Strand Conformational Polymorphism, and automatic sequencing).

Results and Discussion

Four new mutations in *exon* 2 of *CDKN1C* gene were described: one mutation (+275C/T) in the control group of pregnant women that promotes the exchange of arginine by cysteine; one +317A/G glutamine>arginine in a female from the general population; and +495 C/G proline>alanine and +498 C/T leucine>phenylalanine in two distinct pregnant women with diagnosed CH. Although this study did not evidence association of variants of the *CDKN1C* gene directly with the HDP, the results shows that this gene may be involved in the development of CH (4.65% of CH cases) and, therefore, should be also assessed in cases of hypertension unrelated to pregnancy.

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Morphology of 50 day postcoitum agouti fetuses

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Introduction

Studies on the animal prenatal development are important for the characterization of species. In wild rodents, researches were performed in *Agouti paca* (1) and in *Myocastor coypus* (2, 3). This study aimed to characterize the morphology of 50 day postcoitum (pc) agouti fetuses (*Dasyprocta prymnolopha*).

Material and Methods

We used one female of agouti raised in the Center for the Study and Preservation of Wild Animals of UFPI (IBAMA 02/08-618). Daily colpocytology was performed to monitor the estrous cycle and identify sperm cells into vagina. The agouti was weighed and submitted to abdominal ultrasound every on 10, 20, 30, 40 and 50 days pc. Two fetuses were collected by cesarean section. Then they were examined, weighed on digital scales and measured with a cotton yarn and digital caliper. The values were expressed as mean ± standard deviation.

Results and Discussion

The fetuses in this study exhibited smooth, transparent and hairless skin, except in the facial region. In the skull we observed the round optic vesicles with pigmented retina; bilateral earlobes; formed and closed nostrils, open mouth with no erupted teeth. In the thoracic region, dark spots indicating the organs (heart and liver), and clear lines indicating the ribs. The limbs were differentiated and no interdigital membrane or nails were observed. In the thoracic limbs there were observed five digits and in the pelvic limbs three digits. In the perianal area we observed the genital tubercle, anal edge and undifferentiated gonada. The small tail observed is a peculiar characteristic of the species (Fig. 1). Morphometric data of the fetuses are shown in Table 1. A series of similarities were observed when we compared the external morphology of agouti and paca fetuses (1) there were observed similarities. Comparing the agoutis' characteristics with those of 60 day pc coypu fetuses, the only difference observed was the presence of interdigital membrane in coypus (3, 4).

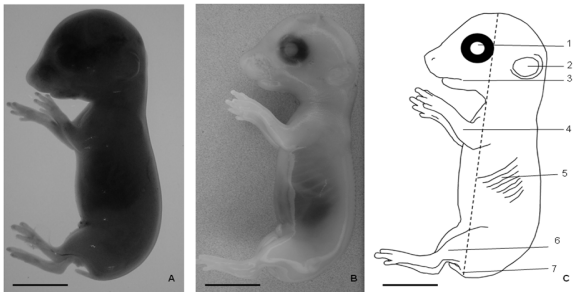

Fig. 1 – 50 day pc agouti fetus (*Dasyprocta prymnolopha)*, lateral view. Fresh fetus (A), buffered formalin fixed fetus (B) e schematic figure (C). Note on the dotted line Crown-Rump lenght; optic vesicle (1), ear (2), mouth (3), thoracic limbs (4), ribs (5), pelvic limbs (6) and tail (7). Bar 1cm.

Table 1. Morphometric data of 50 days pc agouti fetuses (*Dasyprocta prymnolopha*). Weight (W) - g, Lenght - cm: crown-rump lenght (CRL), total lenght (TL), cephalo-caudal length (CCL), cephalic length (CL), Tail length (Tl), eyes (Ey), ear (Ea), thoracic perimeter (TP), abdominal perimeter (AP), biparietal diameter (DBP) and tíbia (TB), Teresina, Piauí, Brazil, 2010.

Obs	W	CRL	TL	CCL	CL	TI	Ey	Ea	TP	AP	DBP	TB
F1	5.2	4.8	7.8	5.9	2.2	0.3	0.6	0.5	3.8	3.9	1.2	0.6
F2	8.9	5.2	8.2	6.4	2.6	0.3	0.6	0.6	3.9	3.8	1.5	0.9
Mean	7.1	5.0	8.0	6.2	2.4	0.3	0.6	0.6	3.9	3.9	1.4	0.8
Standard	2.62	0.28	0.28	0.35	0.28	0.00	0.00	0.07	0.07	0.07	0.21	0.21

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Embryo production and GLUT-1 expression in superovulated goats fed with detoxified castor bean meal

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Introduction

Detoxified castor bean meal (DCBM) is a byproduct of Biodiesel industry and represents an important alternative protein source for animal feeding in arid areas as Northeast of Brazil. Effect of DCBM on productive responses is well documented (1); however there is no information on reproductive toxicity. Thus the aim of this work was to investigate the effect of DCBM on embryo production in goats.

Materials and Methods

Twenty multiparous adult goats were fed to provide 150% of energy requirements, with Bermuda hay plus concentrate where the protein source was soybean meal (WCM group; n = 10) or detoxified castor bean meal (DCM group; n = 10). Effective detoxification in castor bean meal was verified by electrophoresis analysis. All females were synchronized/superovulated with MPA sponge, cloprostenol and pFSH in accordance with (2). Embryos were collected seven days after mating and classified using IETS guidelines. From each fed group an embryo pool (10 embryos/pool) was submitted to the expression of GLUT-1 gene by RT-PCR procedure. The intensity of each band was assessed by densitometric analysis performed with the Image J software. Differences among embryo recovery were analyzed by Mann-Whitney U test for two independent groups. Values were expressed as mean ± SEM.

Results and Discussion

Goats from groups fed with DCBM showed a significant decrease ($p < 0.05$) of embryo flushed from right uterine horn (Fig. 1). By contrast left embryo recovery exhibited similar means values ($p > 0.05$). Although feed treatment did not affect the degenerated embryos rate (14.24 ± 7.93% pooled mean), in the WCM group the frequency of total embryos yielded was higher than the goats fed with DCBM (11.60 ± 1.32 vs. 7.11 ± 2.32; $p > 0.05$) and similar to response reported by (3). Furthermore, in the DCM group the expression of GLUT-1 was inferior to WCM goats (Fig. 2). Results indicate that DCBM can exert an influence on embryo production in goats

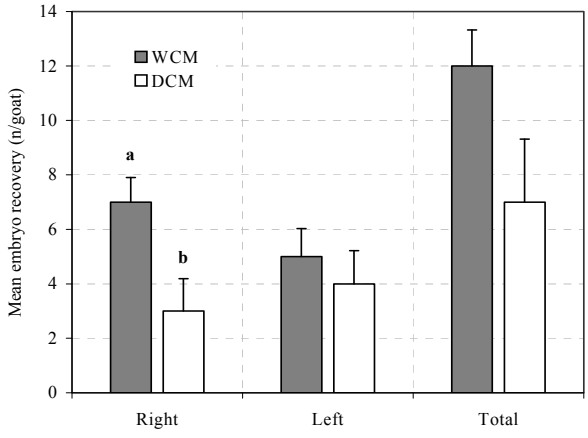

Figure 1. Mean ± SEM embryo recovery

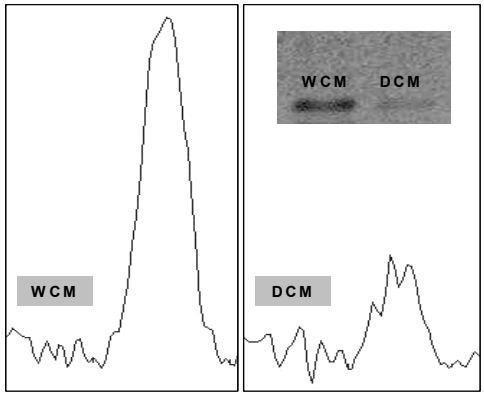

Figure 2. GLUT-1 embryo gene expression

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TSPX and *SRY* expression in bovine preimplantation embryos

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Introduction

TSPX and *SRY* are X and Y chromosomes specific genes, respectively. They are involved in transcription, cell cycle regulation and could be related to differences of development between female and male mammal embryos. Objectives: To verify by conventional PCR the *TSPX* and *SRY'*s pattern of expression in bovine preimplantation embryos produced by *in vitro* fertilization and to compare *TSPX* expression between male and female embryos by real time PCR.

Material and Methods

Bovine testis was used as a control sample, and 20 preimplantation embryos [1-2 (24 h post fertilization); 2-4 (48 h post fertilization); 4-8 (72 h post fertilization); 8-16 (96 h post fertilization); 16-32(120 h post fertilization) cells, morulae (144 h post fertilization) and blastocyst (168 h post fertilization) stages] were pooled and collected. Five 5 IVF replicates were conducted and embryos were collected from all the stages within each IVF replicate to verify the gene expression of *SRY* and *TSPX* by conventional PCR. For real time PCR experiments, three replicates were performed from each sample, six single embryos (normal blastoyst stage) were submitted to the concomitant extraction of DNA and RNA. The DNA was used for embryo sexing by PCR amplification of the *TSPY* gene as a male specific marker. The *β-actin* gene was used as housekeeping gene.

Results

TSPX and *SRY* were expressed in testicular tissue and in bovine preimplantation embryo pools except in embryos at 1-2 cell stage. The real time PCR assay for single sexed blastocysts showed *TSPX* transcripts only in female embryos.

Conclusion

The genes studied showed expression in all stages, except at 1-2 cells. The differential *TSPX* expression pattern between males and females detected at the blastocyst stage, associated to male-specific *SRY* expression, could contribute to different speeds of development in male and female embryos, reported by several researchers.

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CDKN1C gene expression in bovine blastocysts and oocytes

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Introduction

Assisted reproductive technologies (ART), such as *in vitro* systems for oocyte maturation, fertilization and embryo culture, may cause adverse consequences in embryos morphology, metabolism and gene expression (1). Disorders of genomic imprinting have been linked to ART and result in alterations on embryo growth and development. The imprinted gene *CDKN1C* (*p57^{kip2}*) is a maternally expressed gene that encodes a cyclin-dependent kinase inhibitor 1C, which works as a negative regulator of cell proliferation Mutations that affect the epigenetic imprinting status in this gene are implicated in cancers and human developmental disorders (2). The aim of this study was to verify the presence of *CDK1C* transcripts in bovine blastocysts and oocytes.

Material and Methods

Metaphase II (MII) oocytes were obtained after *in vitro* maturation in culture medium TCM199^{+} and cumulus cells were removed with 0.2% hyaluronidase and pooled in 25 oocytes per sample. Blastocysts were acquired after *in vitro* fertilization with FERT-TALP medium. Pooled oocytes and single blastocysts RNA extraction was performed with Allprep DNA/RNA Micro-Kit (Qiagen) and the cDNA was synthesized using Super-ScriptIII RnaseH ReverseTranscriptase (Invitrogen). The qualitative PCR was performed to detect the presence or absence of *CDKN1C* transcripts, and *β-actin* gene was used as an endogenous control. As a comparative gene expression in oocytes, we used the *LIT1* (*KCNQ1OT1*) gene, an antisense transcript.

Results and Discussion

The presence of *CDKN1C* transcripts was found in both oocytes and blastocysts and the quantification of the gene expression will be carried out by real time PCR to verify differential expression patterns. The presence of *LIT1* transcritpts was observed in two MII pooled oocytes. This is the first report about *CDKN1C* gene expression in bovine preimplantation embryos, a gene that is very important to embryo growth and development.

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A novel, objective methodology for gross morphological classification of placentomes in the bovine placenta

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Introduction

Evaluation of placental morphology is a complex and important subject. Normal placentome growth and development is essential for normal fetal growth and development (1). However assessment of gross morphological abnormality on placentome is usually performed by visual, non-objective parameters (1, 2). Objective was to measure numbers of placentomes according to an objective method of gross morphological classification.

Material and Methods

Twenty four bovine pregnant uteri were obtained from a slaughterhouse in Sao Jose dos Campos, SP. Sex of the fetus was established by inspection. Gestational age was estimated using crown-rump (CR) length applied to Richardson´s (3) equation CR = 2.5 (Y + 21). Location of the placentome was established as being in the pregnant uterine horn or non-pregnant uterine horn (1). Measurements were taken in centimeters up to the nearest mm using a metal pachymeter. The following measurements were taken from each individual placentome: the flat length of the longest axis (length), the flat length of the perpendicular axis to the longest one (width) and the height. To assess shape, measures of length were divided by width of each placentome. To assess type, measures of width were divided by height of each placentome. Placentomes were classified according to shape (Table 1) or type (Table 2).

Table 1. Parameters for classification of placentomes according to shape.

Shape	Ratio between measures
Round	Bigger or equal to 1 and smaller than 1.25
Elliptic	Bigger to 1.25 and smaller or than 1.75
Elongated	Bigger than 1.75

Table 2. Parameters for classification of placentomes according to type.

Type	Ratio between measures
Squared	Bigger or equal to 1 and smaller than 2.1
Rectangular	Bigger or equal to 2.1 and smaller than 4.1
Flat	Bigger or equal to 4.1

All results were evaluated using the software R, version 2.7.2 nlme' with the package (Linear and Nonlinear Mixed Effects Models), function 'lme' (Linear Mixed-Effects Models) for ANOVA.

Results and Discussion

A total of 1992 placentomes were evaluated. Numbers of placentomes according to shape nor type were not influenced by stage of gestation, sex of fetus or uterine horn ($p > 0.05$; Table 3).

Table 3. Mean and Standard deviation of the number of placentomes allocated for shape and type.

Shape	Mean	SD	Type	Mean	SD
Round	24.03	14.81	Squared	33.06	17.47
Elliptic	41.50	12.40	Rectangular	51.33	18.86
Elongated	25.71	15.09	Flat	6.92	8.84

This is the first report of an objective methodology to assess placentome gross morphological characteristics. This methodology allows safer comparison between bovine placental data produced by different researcher´s as it is an objective methodology, highly repeatable, that can be performed with minimum training.

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Effects of different extenders on sperm cryocapacitation in Nelore bulls

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Introduction

Extenders containing egg yolk are used in bovine semen cryopreservation, however several arguments exist against their use, among others: great composition variability, microbial contamination risk and exotic disease introduction when the commercialization involves different countries. Egg yolk cryoprotective properties are attributable to phosphatidilcoline (lecithin), phospholipids and lipoproteins. There is consensus that lecithin provides sperm protection during cold shock and freezing. Therefore, soy lecithin based extenders are a plausible alternative for substituting animal derived lecithin. (1). Some authors pose the hypothesis that cryopreservation promotes sperm capacitation. Thus, this study aimed to evaluate the effects of sperm cryo-capacitation during cryopreservation using two different extenders in Nelore bulls.

Material and Methods

Six semen collections were performed in 10 Nelore bulls. Fresh semen was evaluated, and then divided in two treatments: one fraction was cryopreserved using egg yolk based extender (Botu-Bov®-DG) and the second using a soy lecithin based extender (AndroMed®- DL). Samples were then submitted to freezing standard protocols. Four straws of semen from each treatment were thawed, and submitted to a Computer-Assisted Semen Analysis (CASA) to evaluate hyperactivated sperm cells using Edit/Sort program with the parameters: curvilinear velocity (VCL $\geq$ 180), linearity (LIN $\leq$ 45), amplitude of lateral head displacement (ALH max > 10) (2). Acrosomal reaction evaluation was performed using the association of propidium iodide, FITC-PSA and H33342 fluorescent probes (3) and the percentage of capacitated cell was evaluated with YoPro-1, Merocianine 540 and H33342 (4). These samples were evaluated by flow cytometry (BD FACSAria®). Variables were submitted to variance analysis and LSD test in order to compare averages, at 5% of significance level.

Results and Discussion

As shown in table 1, the results from the current study showed that thawed semen has distinct subpopulations, containing capacitated and hyperactivated cells. During the freezing and thawing process, samples from DL group had a greater population of hyperactivated cells ($p < 0.05$) and acrosome reacted cells ($p < 0.05$). Sperm population considered non-capacitated was greater ($p < 0.05$) for the DG group. Based on these results, it can be concluded that soy lecithin based extender causes more cryo-capacitation in sperm cells than egg yolk based extender.

Table 1. CASA and FC characteristics for two different extenders after cryopreservation. Data are presented as mean ± SEM.

Variables	Extenders	
	DG (%)	DL (%)
Hyperactivated cells	6.66 ± 0.67^{b}	12.50 ± 1.02^{a}
Acrosome reacted	32.58 ± 1.42^{b}	50.78 ± 2.71^{a}
Non-capacitated viable cells	14.22 ± 1.13^{a}	6.81 ± 0.73^{b}

Different letters in the same row indicate statistical difference
DG –Botu-Bov® Extender, egg yolk based.
DL –AndroMed®Extender, soy lecithin based.

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Preservation of equine preantral follicles in PBS and MEM – preliminary results

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Introduction

The development of protocols for preservation of preantral follicles is the first step to obtaining and handling the thousands of follicles included in mammalian ovaries, dead or alive. The transport condition to the laboratory is crucial to the success of applying other biotechnologies to the female gametes. The aim of this study was to evaluate the impact of temperature, culture media and time post-harvest on the viability of preantral follicles preserved in situ in the mare.

Materials and Methods

Ovaries (n = 10) from mares in sazonal anestrus (n = 10) of various breeds were obtained in a horse slaughterhouse, 120 km far away from the laboratory. Immediately after collection, ovaries were washed in 70% alcohol and 0.9% saline solution for 10 seconds and then dissected by dividing the cortical portion in 19 fragments of approximately 3 x 3 x 1 mm. A fragment of each ovary was immediately fixed in Carnoy solution for 24 h as control (T1). The remaining 18 were distributed in tubes containing 10 ml of MEM (Minimum Essential Medium) or PBS (Phosphate-Buffered Saline) at 4, 20 or 39°C, and conserved for 4, 12 or 24 hours. The evaluation was performed in all treatments by standard histology and staining with periodic acid Schiff (PAS) - hematoxylin.

Results and Discussion

A preliminary analysis showed that temperature seems to be more important to maintain the follicular viability than media of preservation. At the temperature of 4 degrees, in both media (MEM or PBS), the follicles were shown to be well preserved, with a tendency toward a higher percentage of degeneration as the time interval increased. The temperature of 4°C probably lowered the cellular metabolism, slowing down the degenerative process. These results are comparable to those reported for sheep (1) and cow (2) preantral follicles stored at the same temperatures. At temperatures of 20ºC and 39ºC a high degree of degeneration at all times and studied media was observed. This reduction of viability could be attributed to a greater sensitivity of oocytes enclosed in preantral follicles to conservation (3). The greatest resistance of oocytes enclosed in antral follicles can be due to the large accumulation of reserves nutrients during the growth phase and follicular maturation. Oocytes included in preantral follicles are quiescent or in early stages of growth, then have low nutrient reserves and may be more sensitive to adverse conditions (4). According to our knowledge, this is the first study to report a viable strategy for the transport of mare ovaries, considering time, media, and temperatures. This may increase the probability of success in the use of thousands primordial follicles of the equine species in promising biotechnologies such as IVF and cloning. Furthermore, it needs to be emphasized that there is a need of better understanding the follicular physiology of equine species, considering the lack of ovaries of mares available for experimental studies. These preliminary results showed that ovarian follicles are morphologically normal when preserved in situ in MEM or PBS at 4°C for 4 hours.

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Viability analysis of sperm mediated gene transfer on bovine sperm cells

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Introduction

Most techniques used to produce transgenic animals are laborious and expensive. In this manner, sperm mediated gene transfer (SMGT) may be a viable alternative for large-scale production of transgenic animals. Many SMGT studies have focused the DNA internalization efficiency and number of DNA copies incorporated by spermatozoa. However, limited data are available about the direct effects of the SMGT technique on sperm cells. Hence, this study verified acrosome and plasmatic membrane integrity and mitochondrial membrane potential of sperm cells subjected to SMGT.

Material and Methods

Thawed semen from 3 different bulls was submitted to a 45-90% Percoll gradient. Viable sperm cells were incubated with recombinant PCX-EGFP vector (0, 250, 500 or 1000 ng/10^6 sperm cells) or incubated and electroporated (300V, 35 µF and 0.25 ms). This procedure was conducted in triplicate using the same semen batch. The mitochondrial membrane potential (MMP), acrosomal membrane (AM) and plasmatic membrane (PM) integrity were assessed by flow cytometry (Guava Technologies, Hayward, CA, USA) using JC1, FITC-PSA and PI probes (Molecular Probes), respectively. Data were analyzed by parametric ANOVA (LSD test) using Statistica for Windows software, at a 5% level.

Results and Discussion

No deleterious effects of exogenous DNA concentrations on PM (Fig. 1) or on MMP (Table 1) were observed. However, the addition of 500 ng of exogenous DNA caused sperm AM injury ($p < 0.05$). This was unexpected because there was no effect of higher or lower concentrations of exogenous DNA. Electroporation did not affect PM or AM integrity, but it had a great effect on MMP, which may cause a reduction of mitochondrial function. This may be related to the high percentage of acrosome-damaged cells that was observed in this study, including the control group (0ng of PCX-EGFP vector). These data suggest that more efforts are needed to elucidate the effects of SMGT in sperm cells.

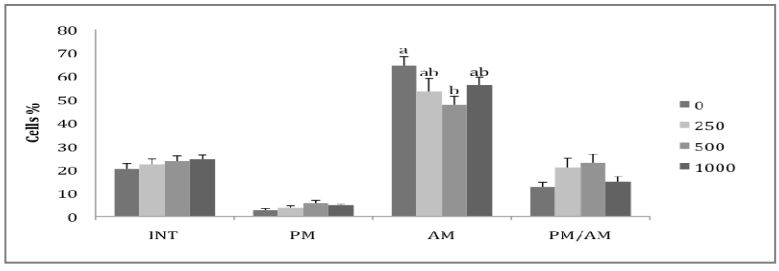

Figure 1. Percentage of intact sperm (INT) with plasma membrane lesions (PM), acrosomal membrane injury (AM) or with both membranes (PM/ AM) injured, incubated with different concentrations of exogenous DNA (LSM ± SE). Lowercase letters (a, b) indicate significant differences within the same variable ($p < 0.05$).

Table 1. Percentages of sperm cells with high mitochondrial membrane potential (MMP) in different exogenous DNA treatments.

	0	250	500	1000
Incubation	$67.40 \pm 1.95^{a, A}$	$62.79 \pm 2.18^{a, A}$	$66.56 \pm 1.17^{a, A}$	$67.03 \pm 0.69^{a, A}$
Eletroporation	$46.16 \pm 3.89^{b, A}$	$55.67 \pm 3.70^{a, B}$	$50.81 \pm 1.78^{b, AB}$	$52.24 \pm 0.87^{b, AB}$

a,b: different lowercase letters indicates differences between columns ($p < 0.05$).
A,B: different uppercase letters indicates differences between lines ($p < 0.05$).

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Recombinant production of a bioactive form of the bovine acidic Seminal Plasma Fluid Protein (aSFP)

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Introduction

The influence of seminal plasma on semen physiology is well known. However, the role of seminal plasma proteins on spermatozoa function and protection during fertilization and cryopreservation is yet to be determined (1). The use of recombinant DNA technology makes possible a deeper investigation on protein structure and function. The present work presents the recombinant expression of the 14 kDa acidic seminal fluid protein (aSFP) associated with bovine semen freezability (2).

Material and Methods

cDNA libraries from bovine prostate, seminal vesicle and bulbouretral gland were constructed, and primers were designed based on data available on Genebank (aSFP: access number NM_174616). Amplicons of the target genes were cloned into pET23a(+), and the construct pET-aSFP was transformed into electrocompetent *E. coli* strains: BL21, BL21 (DE3), BL21 Star, BL21 Codon Plus e BL21 pLysS. For the expression trials, different concentrations of IPTG (***Isopropyl***-beta-D-thio-***galactopyranoside***) were tested, as well several different expression induction conditions. The recombinant expression was analyzed by SDS-PAGE and western blotting. The purification of the target protein was performed by immobilized metal ion chromatography. The bioactivity of the recombinant aSFP (*r*aSFP-*6x*His) was accessed by its ability to bind sperm (3).

Results and Discussion

The best expression for aSFP*r-6x*His (15 kDa) was obtained with the strain BL21 pLysS, induced with 0.5 mM IPTG in 3 hours at 37°C. The target protein was purified by immobilized metal ion chromatography in denaturing conditions with a yield of 2.5 mg/mL per 10 mL of bacterial culture. Western blotting analysis showed the ability of *r*aSFP-*6x*His to bind sperm. The production of seminal plasma recombinant proteins is a new tool for the study of its functions. The improvement of expression and purification processes is important for this biotechnology which has a great potential for therapeutic and diagnose applications.

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Oocyte meiotic resumption induced by angiotensin II is mediated by progesterone

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Introduction

The oocyte meiotic resumption is triggered by the ovulatory gonadotropin surge. Our previous data suggest that both angiotensin II (AngII) and progesterone (P_4) are key mediators of this gonadotropin induced event in bovines (1, 2). Also, these factors appear to be intermediates in the prostaglandin pathway to induce meiotic resumption in bovine oocytes. Therefore, we tested the hypothesis that AngII is an upstream factor to P4 on the gonadotropin induced nuclear maturation.

Materials and Methods

The experiment was performed in three replications. Cumulus-oocyte complexes (COC; n = 540) from cow ovaries (collected at a local abattoir) were selected and distributed among the following seven groups for culture in 200µl of semi-defined media (TCM 199 + 0.4% BSA, 5 µg/ml of LH, 0.5 µg/ml of FSH, 0.2 mM of Pyruvate and 100IU/ml of penicillin/streptomycin): positive and negative controls; AngII (10^{-11}M); AngII plus Mifepristone (MIFE; 1µM; P4 antagonist); P4 (100 ng/ml), P4 plus saralasin (10^{-5}M; AngII antagonist): and AngII plus saralasin. In all treatment groups and in the negative control group, follicular hemisections (2 per 50 µl of media) and COCs (~25/replication) were co-cultured. After 15 h of culture, oocytes were denuded, fixed with triton 100x, and classified according to their nuclear chromatin configuration using a fluorescent microscope (Hoechst staining). Oocytes in metaphase I or latter stages were considered to have a normal resumption of meiosis.

Results and Discussion

The resumption of meiosis induced by AngII and P4 was in a higher rate than in the negative control group (Fig 1; $p < 0.01$). Mifepristone and saralasin inhibited the AngII stimulated effect ($p < 0.01$). However, saralasin had no effect on the meiotic resumption when P4 was present in the culture system. These results are in agreement with our hypothesis that AngII comes before progesterone on the pathway of the oocyte nuclear maturation induced by the pre-ovulatory LH surge.

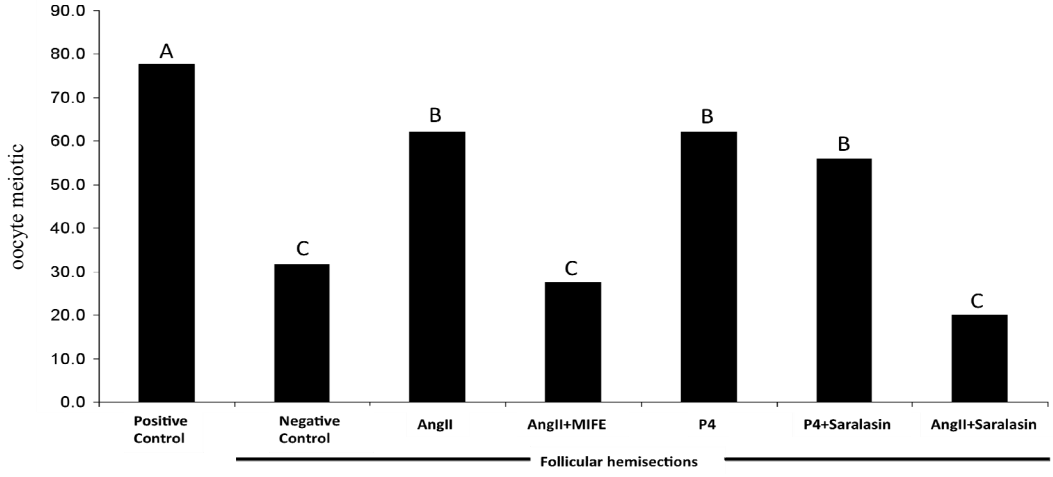

Figure 1. **A)** Oocyte meiotic resumption (metaphase I or latter stages) after 15 h of co-culture with follicular hemisections and different treatments. Significant differences ($P < 0.01$) are indicated by different letters.

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Study of the composition of protein seminal plasma equines during the reproductive period (2004 to 2005)

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Introduction

Spermatozoa acquire the ability to fertilize an egg during a complex, sequentially ordered process known as post-testicular sperm maturation (1, 2). Some seminal proteins are important and natural tools for regulating the physiology activity of the fertilization (3, 4). Studies of proteins in seminal plasma have become useful in understanding mechanisms related to the process of fertilization and cryopreservation of sperm cells. The current study has the objective to evaluate the concentration of stallion seminal plasma proteins, during September 2004 through March 2005.

Materials and Methods

Animals. Ejaculates from eleven stallions (5-20 years old) were collected using an artificial vagina (model "Hannover"). Ejaculates containing a minimum of 50% of spermatozoa with progressive motility were used in this study. All samples were cooled to +5ºC with a cooling rate of 1°C/min. *Protein purification.* In order to obtain seminal plasma proteins, semen was centrifuged for 30 min at +4ºC (600 × g). Then, the supernatant was brought to 36% (wt/vol) saturation with solid ammonium sulphate adjusted to pH 2.0 with 6N HCl, stirred for 30 min, and allowed to stand at 0ºC for 30 min. The samples were centrifuged again (600 × g, 4ºC, 30 min), and the supernatant dialyzed for 48 h in several volumes of 0.5% acetic acid using exclusion membrane 1000 Da, and then lyophilized. Proteins were further purified by gel filtration chromatography using a Superose 12 HR 10/30 column equilibrated with 25 mM Tris- HCl, pH 7.4 in a fast performance liquid chromatography (FPLC-system) (4). Protein concentration was measured with Coomassie Brilliant Blue BG-250 (5), before and after chromatography assay. *Proteinase inhibitory activity.* Trypsin activity was used to evaluate proteinase inhibitory activity. The trypsin activity was assessed spectrophotometrically at 410 nm using a synthetic substrate N-alpha-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) in 25 mM (Tris-HCl), pH 7.4 at 37ºC.

Results and Discussion

In the present study, a negative regression between stallion age and semen supernatant protein concentration was found. A difference in the concentration of different proteins fractions related to the collection period was noticed as well. The average concentration of total protein of ejaculates was 3.39 mg / ml. The mean and standard deviation of the proteins of the eleven animals are represented by retention times, by chromatography, featuring the four main points of molecular weights. We observed a change in protein concentration between ejaculates, especially among the retention times of 14.76 ± 0.21 and 40.81 ± 1.15 min, suggesting an inverse relationship between protein concentrations at these two retention times. It is suggested that this variation may be caused by external and internal variables. This study of stallion seminal plasma protein, specially the serine proteases Inhibitors had more relation with animal ages and can be a tool for understanding the mechanism of fertility as well as being a biomarker discovery.

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Comparison between freezing methods for cryopreservation of stallion spermatozoa

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Introduction

Optimizing cryopreservation of equine sperm will facilitate genetic banking and propagation of important horse strains through assisted reproduction. However, effective and widespread use of frozen equine sperm is still limited, mainly because of its association with low fertility and inconsistent results. The aim of the present study was to compare the post-thaw function of stallion spermatozoa frozen in 0.5 or 0.25 mL straws in either liquid nitrogen vapour (Styrofoam® box) or two programmable freezing machine (TK® 4000C vs Mini Digitcool®)

Material and methods

For this experiment, three ejaculates from four stallions with known fertility were used. The ejaculates were filtered and submitted to analysis by CASA (HTM – IVOS 12, Hamilton Thorne Research, USA). In addition the plasma membrane integrity was determined by fluorescent probes. After evaluation, the ejaculates were diluted at 1:1 (extender: semen) with a skim milk extender Botu-Semen™ and centrifuged at 600 x g for 10 min. The supernatant was removed and the pellet re-suspended to a final concentration of 100×10^6 sperm/ml with milk-egg yolk freezing extender (Botu-Crio™2). Semen was packaged in 0.5 ml and 0.25 ml straws (IMV, L'Aigle, France) and were cooled for 20 min from room temperature to 5ºC and then frozen at a three different cooling systems: protocol 1- programmable freezing machine Mini Digitcool® ZH 400 IMV, L'Aigle (40ºC/min); protocol 2- programmable freezing machine TK® 4000C (40ºC/min); protocol 3- Styrofoam® box (10ºC/min) were placed upon racks suspended 6 cm above liquid nitrogen or other 20 minutes then immersed into nitrogen. All straws were stored in liquid nitrogen until thawing and analysis. The straws were thawed in a water bath at 46◦C for 20 s to straw of 0.5 ml and 46ºC for 12 s to straw of 0.25 ml and the samples were evaluated by CASA and plasma membrane integrity by fluorescent probes. All parameters were analyzed by GraphPad InStat Version 3.06, through unpaired *t* test to identify the significant differences ($P < 0.005$).

Results and Discussion

After post-thaw, there were significant differences in freezing rate between the Styrofoam® box and the programmable freezer TK® 4000C for 0.5 ml straws of total motility (%) and rapid sperms (%; $P < 0.005$). However, no differences were detectable between the three treatments of 0.25ml straws. No deleterious effects were observed after freezing stallion semen in either 0.25 or 0.5 ml straws in the different protocols. For freezing of stallion spermatozoa, the use of 0.5ml has been reported to improve post-thaw sperm parameters in comparison with 0.25ml straws. Clulow (1) hypothesized that the differences in surface area to volume ratio between the two sizes of straw resulted in a slower freezing rate for the larger 0.5 ml straws which was beneficial for ram spermatozoa (1). Cochran (2) observed that stallion spermatozoa tolerated a wide range of freezing rates, suggesting that the small difference in freezing rate between 0.25 and 0.5 ml straws in the post-thaw survival of stallion spermatozoa, supporting the results of the present study (2).

Table 1. Means and standard deviations of sperm parameters using different cooling and freezing package.

Sperm parameters	Styrofoam® Box	Mini Digitcool® ZH 400	TK® 4000C
Total motility (%)			
0.5 ml	65.74 ± 18.63 B	70.68 ± 10.74	74.35 ± 11.79 A
0.25 ml	58.53 ± 16.39	61.63 ± 14.17	63.26 ± 17.21
Progressive motility (%)			
0.5 ml	27.68 ± 11.57	30.73 ± 10.13	31.37 ± 9.34
0.25 ml	23.63 ± 10.18	24,21 ± 9.97	30.74 ± 10.13
Rapid sperm (%)			
0.5 ml	49.95 ± 19.9 B	54.84 ± 16.26	57.89 ± 17.50 A
0.25 ml	42.37 ± 18.09	44.79 ± 17.33	48.47 ± 20.38
Plasma membrane integrity (%)			
0.5 ml	39.37 ± 15.45	38.37 ± 12.92	38.68 ± 13.05
0.25 ml	33.26 ± 13.59	33.84 ± 14.9	34.63 ± 13.03

Different upper case letters (AB) within the same column differ ($P < 0.005$).

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Effects of post-thaw addition of seminal plasma on equine semen: CASA and flow cytometry approach

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Introduction

It is not well known if the addition of seminal plasma after thawing causes damage or improves the viability of the equine spermatozoa. Thus, this experiment was designed with the objective of determining the effect of adding either autologous or homologous seminal plasma (from a stallion with good freezability) to frozen-thawed semen on motility, plasma and acrossomal membrane integrity and mitochondrial function using objective and accurate methods, such as Computer-Assisted Semen Analysis (CASA) and flow cytometry (FC).

Materials and Methods

Five ejaculates of 4 stallions with proven fertility were collected and cryopreserved. The homologous seminal plasma was obtained from a stallion with pregnancy rate higher than 50% per cycle using frozen semen. Autologous seminal plasma was obtained from previous collections from the four stallions that participated in the experiment. The frozen-thawed semen was split into three treatment groups: control: without seminal plasma addition (CON); addition of 20% homologous seminal plasma (HSP); or addition of 20% autologous seminal plasma (ASP). The following variables were analyzed by CASA: total motility (TM, %), progressive motility (PROM, %), amplitude of lateral head displacement (ALH, μm), straightness (STR, %) and linearity (LIN, %). Simultaneous assessments of cells with plasma and acrosomal membrane integrity (IAMI) and with high mitochondrial function (WP) were assessed by FC (BD FACSAria®). Data were submitted to analysis of variance and means were separated by the LSD test.

Results and Discussion

As seen in table 1, the addition of 20% of seminal plasma led to an increase in the IAIM cell population ($P < 0.05$). The addition of seminal plasma did not alter TM ($P > 0.05$), nor the amount of cells with WP ($P > 0.05$). However, it reduced the proportion of cells with PROM ($P < 0.05$), besides reducing LIN and STR. These results together with the increase of the ALH, caused by the addition of autologous seminal plasma, can suggest a possible induction of hypermotility. Thus, with the results found in the present study we discussed that addition of seminal plasma into the frozen-thawed semen before insemination could increase semen fertility by improving the IAIM cell population. Nevertheless, due to the fact that only four stallions were used for this trial and the lack of a fertility test, this experiment can be seen as a pilot study leading to a further large scale study.

Table 1. Sperm plasma and acrosomal membrane integrity, mitochondrial function and motility parameters of frozen-thawed equine semen after division of treatments, as follow: CON, adding freezing extender without a cryoprotectant; ASP, adding autologous seminal plasma; HSP-adding homologous seminal plasma. Data are presented as mean ± SEM.

	Treatment		
Sperm characteristics	COM	ASP	HSP
cells with plasma and acrosomal membrane integrity (%)	31.4 ± 1.2 b	34.2 ± 0.9 a	34.6 ± 1.0 a
cells with high mitochondrial function (%)	31.5 ± 1.1	31.4 ± 1.1	32.6 ± 1.3
total motility (%)	57.7 ± 5.5	58.0 ± 5.9	57.7 ± 5.9
progessive motility (%)	45.3 ± 5.3 a	42.3 ± 5.2 a,b	40.6 ± 5.2 b
amplitude of lateral head displacement (μm)	6.2 ± 0.2 b	6.6 ± 0.3 a	6.5 ± 0.3 a,b
straightness (%)	83.0 ± 0.7 a	80.1 ± 1.1 b	79.1 ± 1.0 b
linearity (%)	48.8 ± 1.2 a	46.4 ± 1-1 b	44.9 ± 1.0 b

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Comparative study of different evaluations of cryopreserved bovine semen imported by Brazil

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Introduction

Artificial insemination is the reproductive biotechnology with the highest contribution to the cattle breeding (1). This would not be possible without the success of sperm cryopreservation. However, cryopreservation causes sperm cell alterations, modifying the general metabolism. The objective of this study was to compare different methods for sperm assessment, in order to find the best strategy of analysis and thus provide reliable parameters for the inspection of bovine semen imported to Brazil.

Materials and Methods

Semen samples from nine Aberdeen Angus bulls were thawed at 36°C/30 sec and the following tests were compared: a-Motility and vigor: subjective assessment *vs* evaluation of membrane potential (JC-1) b-Morphology: Microscopy brightfield with Rose Bengal (RB) *vs* Trypan-Blue and Giemsa (TBG) *vs* Phase contrast microscopy, c-Thermo-resistance test (TRT): Fast TRT (46°C/30 min) *vs* Physiological TRT (36°C/3 h) *vs* Slow TRT (38°C/5 h), d-Integrity of plasm membrane (IPM): hypoosmotic test (HOS) *vs* Eosin-nigrosin (EN) *vs* fluorescent probes (IP-FDA), e- Acrosome integrity: Brightfield microscopy by double staining with TBG *vs* fluorescent probes (PNA-FITC-IP).

Results and Discussion

The average motility of thawed samples was 68.40 ± 7.39%, considered high for cryopreserved semen. However, only 21.00 ± 13.50% of the sperm had high mitochondrial potential, without correlation ((r) = 0.1731) between motility and the high mitochondrial potential.The slow TRT results showed motility and vigor of 12.10 ± 8.87% and 0.68 ± 0.85, respectively, lower than other methods for the same samples, which showed 33.25 ± 12.57% and 2.31 ± 0.48 for the fast TRT and 18.35 ± 11.69% and 1.74 ± 0.49 for the physiological TRT. These results indicate that slow TRT compromises the parameters of motility and vigor. On the evaluation of sperm morphology there was not significant difference between the PC and RB methods. The TBG method identified more abnormalities in the midpiece and tail than the other methods (Table 1), possibly due to its mode of preparation.

Table 1. Sperm morphology by the methods of RB, PC and TBG

Abnormalities	Rose Bengal	Phase Contrast	Trypan Blue and Giemsa
Head	10.27 ± 8.21[a]	11.33 ± 9.60[a]	8.05 ± 4.56[a]
Midpiece	1.77 ± 2.18[a]	2.33 ± 1.50[a]	6.38 ± 3.99[b]
Tail	14.00 ± 4.29[a]	12.16 ± 5.08[a]	27.55 ± 5.11[b]
Total	26.05 ± 11.99[a]	25.83 ± 10.43[a]	42.00 ± 5.23[b]

In rows, values without the same letter (a, b) were different ($P < 0.05$).

The HOS and IP-FDA tests identified significantly fewer sperm with plasm membrane integrity than EN test (HOS: 38.94 ± 14.58; IP-FDA: 46.38 ± 17.29; EN: 64.77 ± 9.64). In the acrosome integrity assessment, there was no significant difference between tests. The TBG and PNA-FITC-IP tests identified that 35.38 ± 11.99% and 31.44 ± 12.07% of sperm presented intact acrosome, respectively. These results indicate that fluorescent probes may be more sensitive than other techniques. In conclusion, the results of this study indicated that for laboratory inspection of bovine semen, the subjective motility, fast TRT or physiological TRT, morphology by PC microscopy, IPM with IP-FDA or HOS, acrosome integrity by PNA-FITC-IP are tests more suitable because of their efficiency and ease of execution.

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Addition of seminal plasma reduces tyrosine phosphorylation in post-thawed equine sperm

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Introduction

The present study investigated the relationship between equine semen preservation and the occurrence of changes similar to capacitation (cryocapacitation) which occur in the spermatozoa during this process and may be related to the increase of lipid peroxidation. Post-thawing addition of autologous seminal plasma or heterologous seminal plasma (from an animal with good semen freezability) was also tested in an attempt to diminish such effects.

Materials and Methods

Five ejaculates from each of 4 stallions with proven fertility were collected, evaluated (raw semen-RS) and the sample was then cryopreserved. The homologous seminal plasma was obtained from a stallion with a pregnancy rate per cycle using frozen semen higher than 50%. Autologous seminal plasma was obtained from previous collections from the four stallions that participated in the experiment. Semen was thawed, evaluated (Post-thawed-PT) and divided into three treatments: no plasma addition (CON), with 20% homologous seminal plasma addition (HSP) or 20% autologous seminal plasma addition (ASP). Semen was incubated in waterbath (37°C) and evaluated at 0,60 and 120 minutes. Membrane disordered - capacitated cells (%), lipid peroxidation (fluorescent intensity, a.u.) and detection of tyrosine phosphorylation on sperm cell surface (Fluorescent intensity, a.u.) were analyzed by flow cytometry (BD FACSAria).

Results and Discussion

Cryopreservation reduced ($P < 0.05$) the population of spermatozoa with plasmatic membrane disordered (RS-9.5 ± 0.7% vs PT-1.6 ± 0.2%). However, it was observed an increase ($P < 0.05$) in membrane peroxidation (RS-90.9 ± 3.7a.u. vs 158.1 ± 14.8a.u.) and induction of protein phosphorylation ($P < 0.05$, RS-76.4 ± 4.1a.u. vs PT-397.5 ± 99.0a.u.). Correlation ($r = 0.73$; $p < 0.05$) was observed between the increase in lipid peroxidation and tyrosine phosphorylation. Seminal plasma, regardless of the source, reduced the population having plasmatic membrane disordered (Fig. 1a) and the quantity of phosphorylated proteins (Fig. 1b). However, it was not capable of reducing lipid peroxidation (Fig. 1c). The present study suggests that the augment of phophorylated proteins on cryopreserved sperm cell surface is a consequence of the increase of lipid peroxidation during the freezing/thawing process. However, the reduction of tyrosine phosphorylation that occurs after seminal plasma addition is triggered by others mechanisms that are independent of membrane peroxidation reduction and were not identifiable in this experiment.

Values with a different letters differ ($P < 0.05$).

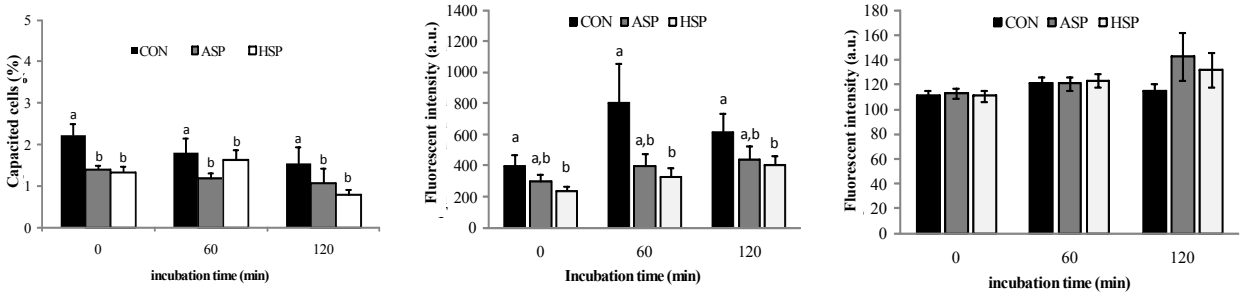

Figure 1. Means ± SEM of (a) capacited cells (%), (b) detection of the tyrosine phosphorylation on sperm cell surface (Fluorescent intensity, a.u.) and (c) lipid peroxidation (fluorescent intensity, a.u.).

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Absence of green fluorescence in *Cumulus oophorus* cells from C57/BL6/EGFP mouse

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Introduction

Generation of animal models, as transgenic mice with enhanced green fluorescent protein (EGFP) is an important tool to understand the morphology and physiology of cells, isolated or in association to other cells. The functional unity of the *cumulus*-oocyte-complex (COC) is of essential importance to oocyte growth, maturation and fertilization understanding. The fluorescence of *cumulus oophorus* cells could be explored (coupled with non fluorescent oocytes) to studies of the influence of those cells on denuded oocytes. The aim of this report was to describe the lack of *cumulus* green fluorescence in COC with fluorescent oocyte from constitutively EGFP mice.

Materials and Methods

Four C57BL/6-Tg (CAG-EGFP) C14-Y01-FM131 Osb females, weighting around 35g were sacrificed to other purposes (*i.e.*, embryo recovery). Ovaries were collected and manipulated in PBS with 0.4% BSA. They were manually fragmented (23G needle) in order to release COCs. After 3 to 4 washes, groups of COCs and eventually non antral follicles were evaluated with bright and UV light, under an inverted microscope (Eclipse T*i*-S, Nikon, Japan). Control COCs from four ovaries (Swiss Webster strain) were used to standardize absence of fluorescence. Images were captured (NIS-Elements Advanced Research, Nikon, Japan) with both light sources and merged.

Results and Discussion

As expected, control COCs did not fluoresce with specific UV light to EGFP (488nm). However, oocytes from C57/BL/6 EGFP fluoresced with high intensity, independently if they came from primordial or antral follicles. Interestingly, *cumulus oophorus* cells did not fluoresce even with their counterpart (*i.e.*, oocyte) strongly glowing. When put together, control COC and C57/BL/6 EGFP COC, no difference was detected between *cumulus oophorus* cells, under UV light. Herein, it was reported that the *cumulus*-oocyte-complex from C57/BL/6 EGFP did not follow the constitutive gene expression of EGFP, as reported previously when the Tg mouse was generated (Okabe *et al.*, 1997). *Cumulus* cells should be put together with hair and red blood cells as the only cells/structures that do not fluoresce on described EGFP mouse.

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Manganese and membrane of spermatozoa in Nellore young bulls

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Introduction

Manganese (Mn) is an essential mineral for cattle and may cause fertility disorders (1). The physiological effects of this mineral and its right dose for Nelore bulls are not clear. This experiment aimed to evaluate the effect of Mn supplementation on plasma membrane integrity spermatozoa of young bulls.

Material and Methods

Sixty Nelore bulls (*Bos indicus*) aged between 16 and 18 months, with the same weight were reared in extensive systems grazing *Brachiaria decumbens* and were divided into four groups (N = 15) supplemented *ad libitum* with mineral mixture containing: Gc=0, G_{1300}=1,300, G_{3800}=3,800 and G_{6300}=6,300 mg Mn/kg for 56 days. Semen was collected from bulls by electroejaculation and spermatozoa plasma membrane integrity was evaluated by the technique of associating fluorescent probes (FITC-PSA, PI, JC-1, H342) (2), sorting the sperm with intact plasma membrane, intact acrosome, with high mitochondrial membrane potential (MPI,AI,APM); intact plasma membrane, intact acrosome, low mitochondrial membrane potential (MPI,AI,BPM); intact plasma membrane, damaged acrosome, high mitochondrial membrane potential (MPI,AL,APM); intact plasma membrane, damaged acrosome, low mitochondrial membrane potential (MPI,AL,BPM); injured plasma membrane, intact acrosome, high mitochondrial membrane potential (MPL,AI,APM); plasma membrane injured, intact acrosome, low mitochondrial membrane potential (MPL,AI,BPM); plasma membrane injured, damaged acrosome, high mitochondrial membrane potential (MPL,AL,APM) and plasma membrane injured, damaged acrosome, low potential mitochondrial membrane (MPL,AL,BP). Data were transformed into arcsine and analyzed by analysis of variance using the procedure GLM of SAS. Means were separated by the Tukey test.

Results and Discussion

All groups showed a high percentage of spermatozoa with injured plasma membrane and low mitochondrial potencial because they were young bulls (Table1). However, bulls of group Gc showed increased percentage ($P < 0.05$) of spermatozoa with intact acrosomal membrane and decreased percentage ($P < 0.05$) of total damaged spermatozoa at day 56 (Table 1), indicating that sperm membrane integrity became better. In contrast, bulls supplemented with Mn at day 56 showed a smaller proportion of ($P < 0.05$) spermatozoa with intact plasma membrane and high mitochondrial potential. Also, percentage of spermatozoa with intact acrosomal membrane were 12.7%; 28.1% and 17.9% smaller ($P < 0.05$) than Gc and G_{3800} bulls had 296.5% more total damaged spermatozoa than Gc (Table 1). In conclusion, Mn supplementation, in the doses tested, was harmful to spermatozoa plasma membrane integrity during the period studied.

Table 1. Spermatozoa classification by fluorescent color of association of probes.

Spermatozoa classification	Days of observation							
	0				56			
	Experimental groups							
	Gc	G_{1300}	G_{3800}	G_{6300}	Gc	G_{1300}	G_{3800}	G_{6300}
MPI, AI, APMM (%)	0.0^{Aa}	0.1^{Aa}	0.1^{Aa}	0.0^{Aa}	0.6^{Aa}	0.0^{Aa}	0.0^{Aa}	0.0^{Aa}
MPI, AI, BPMM (%)	0.0^{Aa}	0.7^{Aa}	0.9^{Aa}	0.0^{Aa}	5.5^{Aa}	0.0^{Aa}	0.0^{Aa}	0.0^{Aa}
MPI, AL, APMM (%)	0.0^{Aa}	0.0^{Aa}	$0.5A^{a}$	$0.0A^{a}$	0.7^{Aa}	0.0^{Aa}	0.0^{Aa}	0.0^{Aa}
MPI, AL, BPMM (%)	0.0^{Aa}	13.1^{Aa}	6.7^{Aa}	0.0^{Aa}	0.0^{Aa}	0.0^{Ab}	0.0^{Ab}	0.0^{Aa}
MPL, AI, APMM (%)	0.0^{Aa}	3.3^{Aa}	8.1^{Aa}	3.3^{Aa}	0.0^{Aa}	0.0^{Ab}	0.0^{Ab}	0.0^{Ab}
MPL, AI, BPMM (%)	44.7^{Aa}	47.1^{Aa}	23.3^{Aa}	44.3^{Aa}	84.8^{Bb}	74.0^{ABb}	61.0^{Ab}	69.6^{ABb}
MPL, AL, APMM (%)	0.0^{Aa}	1.5^{Aa}	1.0^{Aa}	0.0^{Aa}	0.0^{Aa}	0.0^{Aa}	5.3^{Aa}	0.0^{Aa}
MPL, AL, BPMM (%)	55.3^{Aa}	34.1^{Aa}	59.5^{Aa}	52.5^{Aa}	8.5^{Ab}	26.0^{Ab}	33.7^{Bb}	30.4^{ABb}

^{A,B}Numbers followed for different big letters show significant difference ($P < 0.05$) between the groups.
^{a,b}Numbers followed for small different letters show significant difference ($P < 0.05$) between the days of observation.

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Evaluations of the cryopreserved imported bovine semen from three artificial insemination companies

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Introduction

The cryopreservation process and/or thawing can to induce many alterations in mammalian spermatozoa, such as the motility decrease and alteration of membranes (1). The objective of this study was to conduct a series of sperm evaluations in imported cryopreserved bovine semen, from three different artificial insemination (AI) companies to determine the quality of genetic material imported by Brazil.

Materials and Methods

Samples of imported cryopreserved semen of nine Aberdeen Angus bulls from three insemination companies (A, B, C) were used. The straws were thawed at 36°C/30 s and the following parameters were evaluated: a-Motility and vigor: triple subjective evaluation; b-Concentration in Neubauer chamber; c-Morphology by phase contrast (PC) microscopy; d- Fast thermoresistance test (TRT) (46°C/30 min); e-Plasm membrane integrity (PMI) by fluorescent probes (PI-FDA); f-Acrossome integrity by fluorescent probes (FITC-PNA-PI).

Results and Discussion

The results of concentration, motility and vigor of thawed samples were considered appropriated and were not different among the companies (Table 1).

Table 1. Comparative evaluations of sperm concentration (x 10^6 cells/mL), motility (%) and vigor (0-5).

Parameters	Company A	Company B	Company C
Concentration	28.66 ± 7.26	30.15 ± 10.44	25.79 ± 11.46
Motility	67.49 ± 8.70	64.44 ± 8.54	73.88 ± 0.96
Vigor	2.88± 0.19	2.99 ± 0.33	3.11 ± 0.19

Table 2. Comparative evaluation of sperm morphology by PC (%).

Abnormalities	Company A	Company B	Company C
Head	18.66 ± 9.43	5.00 ± 2.64	10.33 ± 11.42
Midpiece	2.83 ± 1.25	1.50 ± 1.32	2.66 ± 2.02
Tail	10.50 ± 1.32	9.66 ± 2.36	16.33 ± 7.52
Total	32.0 ± 9.50	16.16 ± 6.21	29.33 ± 9.54

The average results of sperm morphology evaluation were not significant different between the AI Companies (Table 2). However, considering the individual results, in Company A one bull was identified with 29% of head defects and in Company C one bull was identified with 23,5% of head defects and other bull with 25% of tail defects. These results indicate the lack of quality in sample, which can lead to losses in artificial insemination. In the TRT, the final motility of Company C was 44,99 ± 6,00%, not differing from Company A (25.27 ± 11.91%), and higher than to Company B (8.22 ± 4.59%). The final motility after TTR from Company B is considered low for a sample of quality. This result may have been influenced by the cryoprotector medium used, because all animals evaluated showed low motility after the TTR. It was observed 43,16 ± 24,60%, 51,16 ± 17,29% and 44,83 ± 15,44% of spermatozoa with intact plasma membrane, respectively for AI Companies A, B and C, not differing significantly among them. The evaluation of acrosome integrity demonstrated that 26,66 ± 6,00%, 37,66 ± 11,15%, and 30,00 ± 18,08% of sperm presented an intact acrosome, respectively for AI Companies A, B and C, not differing significantly among them. These results indicate that cryopreservation process used for these AI companies preserved sufficiently the sperm plasma membrane and the acrossomal structure.

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Effects of organic selenium supplementation on motility of boar sperm

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Introduction

Selenium (Se) has an important role in animal reproduction in form of selenoproteins. The Phospholipid Hydroperoxide Glutathione Peroxidase, PHGPx, is found for about 50% of the capsule material, it seems likely that it is this polymerization that confers the structural integrity required for sperm stability and motility (1). Thus, the objective was to evaluate the effects of dietary organic selenium on sperm motility in boar semen stored for 3 days by Computer-Assisted Semen Analysis (CASA).

Materials and Methods

The twelve boars were fed with three experimental diets: 0.3 ppm sodium selenite (Control); 0.5ppm sodium selenite (Inorganic) and 0.5 ppm Se yeast (Organic). From the beginning up to the 6th semen collection, animals were submitted to fortnightly examinations that included a seminal analysis by CASA. Semen was diluted with standard extender to $3x10^9$ cells/dose and stored for 3 days at 18ºC. The following variables were analyzed by CASA: total motility (TM), progressive motility (PROM), amplitude of lateral head displacement (ALH), straightness (STR) and linearity (LIN). Data were analyzed using REPEATED command, generated by the PROC GLM from SAS and outcomes were compared among the three treatments by the orthogonal contrast method with $P < 0.05$ level of significance.

Results and Discussion

There was no significant interaction between treatments and storage period for any of the sperm motility parameters assessed (Table 1). For the period, TM and PROM showed greater ($P < 0.05$) reduction from 48 hours after dilution. There was effect ($P < 0.05$) of Se source for the characteristics TM and PROM, finding higher percentages for the organic source compared to the inorganic. The characteristics ALH, STR and LIN declined ($P < 0.05$) with increasing storage time. Moreover, the STR and LIN differed ($P < 0.05$) in relation to supplementation, males fed 0.3 ppm sodium selenite had higher percentages compared to other treatments. In conclusion, the use of 0.5ppm Se yeast seems able to improve the total and progressive motility.

Table 1- Sperm motility parameters obtained by CASA of storage boar semen.

Semen Characteristics	Semen Storage							
	Treatments (ppm)				Probability			
	Se Inorganic		Se Organic	SEM	Contrasts		P	T*P
	0.3	0.5	0.5		C1	C2		
TM (%)	81.95	79.88	84.41	0.75	ns	<.05	<0.05	ns
PROM (%)	40.96	35.73	44.27	1.14	ns	<.05	<0.05	ns
ALH (μm)	5.83	5.98	6.08	0.07	ns	ns	<0.05	ns
STR (%)	71.97	68.58	69.84	0.49	<0.05	ns	<0.05	ns
LIN (%)	40.45	37.33	39.40	0.48	<0.05	<0.10	<0.05	ns

C1 (Contrast 1) = 0,3vs0,5ppm Se; C2 (Contrast 2)=0,5ppm Se inorganic vs Se organic; P–period; T*P–treatment*period.

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Effects of shade availability in the pasture on reproductive traits of Nelore bulls (*Bos indicus*) raised in Southeastern region of Brazil

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Introduction

In open environments, great part of body temperature elevation is due to direct incidence of solar radiation. An alternative in order to decrease solar radiation, minimizing heat stress, is the use of artificial shade (1). Studies up to now with Zebu cattle were performed with the objective of comparing its adaptability to tropical climate with European breeds. Hitherto, an effect of heat stress reduction on the reproductive characteristics of Zebu bulls was never tested. Thus, this study aimed to evaluate the effect of thermal stress reduction, through shade availability in paddocks on reproductive characteristics of Nelore bulls (*Bos indicus*).

Material and Methods

Ten bulls were divided as follows: AS= with available artificial shade (n = 5) and US= unavailable artificial shade (n = 5). Each group was kept in two hectares paddocks, in which shade availability for group AS was artificially created. Animals were submitted to a clinical-reproductive evaluation and semen analyses every other week for a total eight times. In the clinical-reproductive examination, testicular consistency score was graded as 1 to 5, with 1 being soft, increasing consistency gradually to a score of 5, corresponding to firm-normal consistency. Sperm motility was assessed by CASA (Hamilton Thorne Biosciences, Ivos-Ultimate, Beverly, MA, USA). The integrity of plasma and acrosomal membranes, as well as mitochondrial function, were evaluated using a combination of propidium iodide, fluorescein isothiocyanate conjugated *Pisum sativum* agglutinin, and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine, iodide fluorescent probes, respectively (2). To assess sperm morphology, semen was diluted and fixated in pre-warmed (37°C) formaldehyde-PBS. Sperm cells (n = 200) were counted under differential interference-contrast microscopy at a magnification of 1000x. Data were submitted to analysis of variance. Results are presented as mean ± S.E.M. and $P < 0.05$ was considered significant.

Results and Discussion

No significant effects ($P > 0.05$) of treatment (AS and US) were observed for any of the variables analyzed. Means for testicular consistency was 3.9 ± 0.1 for the group AS and 3.7 ± 0.1 for the group US. Scrotal circumference means was 30.5 ± 0.3 cm for AS group and 31.7 ± 0.2 for US group. Mean progressive motility and percentage of rapidly moving cells in semen samples were 77.7 ± 1.3% and 86.6 ± 1.3%, respectively for AS group. For the US group mean progressive motility was 79.7 ± 1.2 % and 88.2 ± 1.2% for percentage of rapidly moving cells. Bulls staying in paddocks with shade had mean 66.8 ± 1.7% of sperm simultaneously with plasma and acrosomal membrane integrity and high mitochondrial potential meanwhile bulls without shade had 70.9 ± 1.2%. Mean major defects, minor defects and total defects in semen samples were 12.8 ± 1.2%, 10.1 ± 1.9 and 23.7 ± 2.3% respectively for AS group. US group mean were 11.3 ± 0.9% for major defects, 5.39 ± 0.7% for minor defects and 16.7 ± 1.2% for total defects. It can be concluded that the absence of shaded areas, during summer does not negatively affect reproductive characteristics such as: scrotal circumference, testicular consistency, progressive motility, percentage of rapidly moving cells (CASA), morphology or sperm viability in Nelore bulls reared in extensive systems located in southeastern region of Brazil.

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Andrological evaluation on two spinal cord injured dogs: a case report

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Introduction

Little information is available about the semen quality in dogs with spinal cord injury (SCI), especially concerning spermatogenesis. A study in dogs revealed that, only after three weeks of injury, semen quality changed, sperm motility and spermatogenesis decreased. Moreover, the study predicted that dogs with maintenance of SCI eventually become oligospermic or azoospermic (1). The aim of this study was to evaluate semen quality of two dogs with long and short post-SCI period.

Material and Methods

Two dogs attending the University Hospital with SCI were selected: one teckel mix (A), 4 years old, body weight 10 kg and 18 months post-injury, with SCI at T12-T13 and L1-L2-L3-L4 level, and one teckel (B), 6 years, body weight 8 kg and 3 months post-injury, with SCI at T11- T12- T13-L1 level. Andrological examinations were performed and one ejaculate from each animal was collected by digital manipulation and semen analyses were performed.

Results and Discussion

Andrological examination of both animals indicated testicular degeneration. Dogs were easily collected by digital manipulation without an estrous teaser bitch. Sperm fractions could not be distinguished and sperm volumes were different for both dogs (Table 1). Dog A was classified as azoospermic. The ejaculate and the bladder content were centrifuged and samples were analyzed with Cerovsky stain. Evaluation of cell morphology was performed at bright field microscopy (Olympus BX61, Japan, 1000x). Multinucleated giant cells, degenerated spermatocytes, spermatids and degenerated cells were observed (Fig. 1). Dog B showed teratospermy, 51% of damaged plasmatic membrane, 100% of low mitochondrial potencial and 100% of chromatin integrity. The spermiogram for dogs A and B were different and may be related to the duration and site of SCI. Additional studies will be conducted considering time post-injury, and local of the SCI versus semen quality in more selected dogs, this may contribute to the understanding of changes in sperm quality.

Table 1. Characteristics from andrological examination of dogs A and B and normal values (2)

Testis	A	B	Dog 10kg[2]	Ejaculate	A	B	Dog 10 kg[2]
Right				Volume	11 ml	0.4 ml	5-10 ml
Height (cm)	2.3	3.4	2.5-2.0	Motility	-	50%	60-70%
Width (cm)	2.9	2.0	1.8-1.4		-	97	450-300
Left				Sperm-concentration ($x10^6$/ml)			
Height	2.3	3.0	-	Total sperm defects (%)	-	94.5	10-25
Width	1.9	2.0	-				

[2]Normal values according to Günzel-Apel (2).

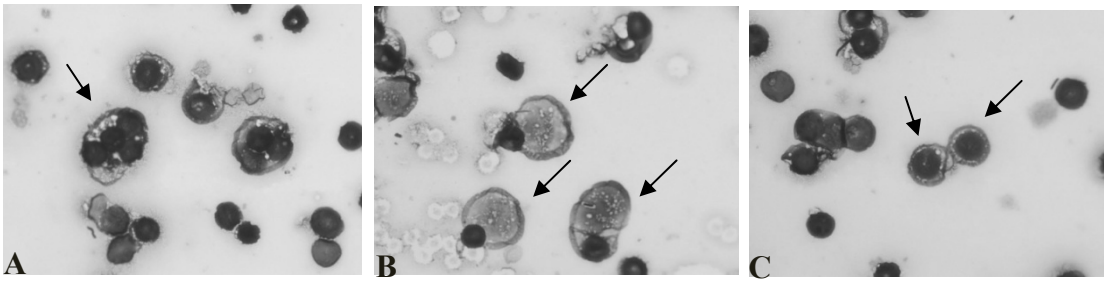

Figure 1. Semen smear of Dog B: A. Multinucleated giant cells; B. Degenerated spermatocyte; C. Spermatid (Cerovsky, 1000x).

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Evaluation of a Nelore bull with high degree of chromatin damage and protamination deficiency - a case report.

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Introduction

Significant progress has been made towards the development of reliable tests for sperm chromatin integrity and DNA damage (1). An important research done by Evenson et al. (2) suggested that assessment of DNA integrity in sperm may be a useful and potentially independent marker of fertility for both animals and men. The aim of this study was to describe and compare the seminal characteristics of a Nelore bull with high levels of chromatin damage.

Material and Methods

Frozen bovine semen samples (three ejaculates) from a sexually mature Nelore bull were received for routine evaluation at our laboratory facilities. All frozen-thawed samples were evaluated for: motility and vigor before and after slow thermo-resistance test (TTR – 37°C/3 hours), percentage of intact acrosome (PIA), morphology, simultaneous evaluation of acrosome, membrane and mitochondrial potential (FITC-PNA, PI, JC-1), chromatin integrity (acridine orange - AO) before and after TRT, and degree of protamination (chromomycin A_3 -CMA_3). Since this animal (Bull A) presented a high percentage of morphological defects (50.83% ± 6.53), mostly related to high percentages of chromatin damage and deficient protamination, we selected him for this case report. Simultaneously, frozen semen samples from five mature Nelore bulls (three ejaculates each) were also selected and used as reference group.

Results and Discussion

No significant differences ($P < 0.05$) between Bull A and the reference group were observed neither on motility and vigor before and after TTR and nor for PIA. Interestingly, Bull A showed greater percentages of morphological sperm defects, chromatin damage before and after TRT and deficient protamination, presented lower values for acrosome and plasma membrane integrity ($P < 0.05$); whereas, mitochondrial status were not different from the reference group (Table 1). Since spermatozoa must possess a large number of attributes in order to fertilize an oocyte, any laboratory measuring a single sperm attribute, such as motility for example, will produce results that do not correlate well with fertility (3). In our case report, Bull A showed the same post-thaw sperm motility as in reference animals, regardless of more morphological defects, chromatin damage and significant levels of protamination deficiency. Thus, measuring multiple attributes seems to be a more appropriate approach to better estimate sperm competence.

Table 1. Ejaculate characteristics of Bull A and the reference group.

	Total sperm defects	Acrossome integrity	Membrane integrity	Mitochondrial potential	Chromatin integrity 0h	Chromatin integrity 3h	Protamine deficiency
Bull A	50.8 ± 6.5*	24.3 ± 3.3*	24.5 ± 6.1*	85.0 ± 2.18	13.8 ± 9.5*	19.8 ± 9.5*	3.7 ± 0.6*
Reference group (n = 5)	4.7 ± 2.7	76.9 ± 8.9	75.7 ± 9.3	83.1 ± 13.3	0.6 ± 0.5	0.6 ± 0.5	0.4 ± 0.6

*($P < 0.05$).
Values are presented as mean ± SEM.

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Evaluation of the bovine spermatozoa from cauda epididymides stored at 5°C for 24 hours, incubated with and without Sperm Talp

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Introduction

Ejaculated sperm differ from epididymal sperm in many factors; include the types of proteins bound to the plasma membrane (1) and different motion characteristics (2). The recovery of viable spermatozoa from cauda epididymis is one important technique for the preservation of genetic of valuable males that have dead. The cryopreservation of epididymal spermatozoa allows a more efficient and economical way to use that material, because it can be utilized anytime not just after death of an animal. An important factor to take into account when this technique is utilized is the conditions under which the testicles were stored, particularly temperature (3). The aim of this study was to evaluate the influence of storage period on motility of bovine sperm harvested from epididymal cauda, incubated with and without Sperm Talp medium.

Materials and Methods

Fifity-six testicles with epididymides (28 bulls) were collected following slaughter, and divided into two groups. One epididymis from each pair was flushed immediately after the slaughter and the other was stored at 5°C for 24 hours, being groups 0 (control) and 24 respectively. The sperm was obtained using retrograde flushing with Botu-semen® (Biotech, Botucatu, São Paulo, Brazil), then the samples were incubated for 15 minutes at 37°C and centrifuged at 2500 rpm/15min with (SP+) or without (SP-) Sperm Talp medium. The samples were evaluated by computer assisted sperm analysis (CASA; HTM—IVOS 12, Hamilton Thorne Research, USA). Statistic Analyses utilized was ANOVA followed by Tukey's test ($P < 0.05$).

Results and Discussion

The samples obtained immediately after the slaughter (control) and incubated with Sperm-Talp (0SP+) presented better result only for progressive motility (PM) that those stored for 24 hours and incubated with Sperm-Talp (24SP+), PM = 51,29 ± 12,61 and 43,07±14,41, respectively. No difference was found between the groups 0 (0SP-) and 24 hours (24SP-) when they were incubated without Sperm-Talp. However, for the epididymides that were stored for 24 hours, the samples incubated with Sperm-Talp (SP+) presented higher values ($P < 0.05$) than without Sperm-Talp (SP-) in some parameters analyzed by CASA: total motility (TM, %) = 24SP- (27.25 ± 18.02) and 24SP+ (71.89 ± 19.06); progressive motility (PM, %) = 24SP- (13.0 ± 9.15) and 24SP+ (43.07 ± 14.41); path velocity (VAP, μm/s) = 24SP- (91.50 ± 24.56) and 24SP+ (126.07 ± 21.98); straight line velocity (VSL, μm/s) = 24SP- (63.36 ± 13.10) and 24SP+ (93.64 ± 16.32); curvilinear velocity (VCL, μm/s) = 24SP- (157.39 ± 35.65) and 24SP+ (216.54 ± 40.72); lateral head displacement (ALH) = 24SP- (6.95±1.55) and 24SP+ (7.99 ± 1.46); beat cross frequency (BCF, Hz) = 24SP- (20.96±4.96) and 24SP+ (30.29±15.31); and percentage of rapid cells (RAP, %) = 24SP- (25.61 ± 20.15) and 24SP+ (69.32 ± 20.26). In addition, the samples from group 0 incubated with Sperm-talp (SP+) also showed better results than without Sperm-Talp (SP-) for some analyzed parameters, such as: (TM, %) = 0SP- (34.04 ± 23.77) and 0SP+ (77.18 ± 17.03); (MP, %) = 0SP- (18.18 ± 11.79) and 0SP+ (51.29 ± 12.61); (VAP, μm/s) = 0SP- (81.14 ± 24.73) and 0SP+ (123.68 ± 27.32); (VSL, μm/s) = 0SP- (58.61±17.22) and 0SP+ (100.18 ± 12.20); (VCL, μm/s) = 0SP- (152.75 ± 43.64) and 0SP+ (217.79 ± 23.70); (BCF, Hz) = 0SP- (22.21 ± 5.36) and 0SP+ (29.89 ± 4.81); Linearity (LIN, %) = 0SP- (38.18 ± 8.04) and 0SP+ (46.86 ± 5.42); (RAP, %) = 0SP- (33.39 ± 23.33) and 0SP+ (74.14 ± 18.11). Based in these results, it was concluded that epididymal sperm incubation with Sperm Talp medium improves the motility parameters and epididymides can be stored for 24 hours at 5°C.

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DNA integrity and protamination *Bos taurus* and *Bos Indicus* bulls - previous note

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Introduction

All sperm processing steps may introduce damage to sperm DNA, membranes and organelles. In order to be able to obtain better fertilization results it is necessary to assess the quality of sperm just before their use. The integrity of sperm DNA can be measured at three different levels by assessing the degree of DNA protamine condensation, the incidence of breaks and nicks in the DNA and the frequency of fragmentation of the nuclei into sub-haploid apoptotic bodies (1). The aim of this study was to evaluate DNA integrity and protamination in cryopreserved semen samples from mature and fertile *Bos indicus* and *Bos taurus* bulls.

Material and Methods

A total of five *Bos indicus* and four *Bos taurus* were selected for this study from an insemination centre. After dilution with egg-yolk-Tris extender, semen was frozen and stored in liquid nitrogen according to pre-established patterns of the artificial insemination centre. Three frozen-thawed batches from each animal were evaluated for motility and vigor before and after slow thermo-resistance test (TRT – 37°C/3 h), percentage of intact acrosome (PIA), total morphological sperm defects (Tot def), DNA integrity (Acridine Orange test – AO) and protamination (Chromomicyn A3 – CMA3).

Results and Discussion

Table 1 summarizes the semen parameters. No differences were observed for *Bos taurus* and *Bos indicus* bulls in this study. The percentage of DNA damage and deficient protamination showed to be very low which is in agreement with the findings of Simões et al. (2), who first described the use of CMA_3 for evaluation of protamination in bovine semen. The very few studies of bovine DNA integrity and protamination claims for further research investment in this area.

Table1. Semen parameters for *Bos indicus* and *Bos taurus* bulls.

	Motility 0h (%)	Motility 3h (%)	Vigor 0h	Vigor 3h	PIA (%)	Tot def (%)	DNA damage (%)	Abnormal protamination (%)
Bos indicus	45.66 ± 4.51	35.32 ± 1.82	4.47 ± 0.07	3.80 ± 0.48	55.26 ± 5.66	10.08 ± 3.26	0.54 ± 0.27	0.40 ± 0.43
Bos taurus	51.25 ± 6.87	38.33 ± 9.53	4.37 ± 0.25	3.70 ± 0,60	57.05 ± 3.86	11.83 ± 3.38	0.55 ± 0.49	0.08 ± 0.22

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Ultrastructural evaluation of acrosomal damage after Percoll gradient centrifugation

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Introduction

Percoll separation methods are in widespread use in bovine *in vitro* fertilization (1, 2) and numerous papers are in agreement that the percentage of spermatozoa presenting plasma membrane integrity is increased after Percoll treatments of frozen-thawed bull semen (1, 2, 3). However, the percentage of sperm cells with intact acrosomal membrane can be reduced (3). The objective of this study was to characterize the ultrastructural acrosome damage after discontinuous Percoll density gradient centrifugation on cryopreserved bovine spermatozoa.

Materials and Methods

Semen was collected from six bulls of different breeds and three ejaculates per bull were evaluated. The discontinuous Percoll density gradient was prepared by layering 2 mL of 90%, 2 mL of 85% and 2 mL of 80% Percoll solutions in 15 mL polystyrene tubes. The frozen semen samples were thawed and the acrosome regions of spermatozoa were evaluated by Transmission Electron Microscopy before (n = 18) and after (n = 18) centrifugation in discontinuous Percoll density gradient. The evaluation of 20 spermatozoa from each of 36 analyzed samples (in all, n = 720) ensured that a sufficiently large number of spermatozoa were investigated. The sperm cells were classified into four different categories: spermatozoa with normal acrosome, spermatozoa presenting "physiological" acrosome reaction (sperm head presenting swelling of acrosomal ground substance with vesicles of fused plasma and outer acrosomal membranes), spermatozoa presenting atypical acrosome reaction (sperm head presenting swelling of acrosomal ground substance dispersed under the swollen outer acrosomal membrane), and spermatozoa with mechanical acrosome injury (sperm head presenting rupture of acrosomal membrane with swelling of acrosomal ground substance in restricted points). The results were analyzed using the SAS program and the variables were submitted to analysis of variance (ANOVA) at a level of significance of 5%.

Results and Discussion

The Percoll gradient centrifugation reduced ($P < 0.01$) the percentage of spermatozoa presenting normal acrosome (from 61.77 ± 2.49% to 30.24 ± 7.78%), reduced ($P < 0.01$) the percentage of spermatozoa presenting atypical acrosome reaction (from 28.38 ± 3.04% to 4.84 ± 3.09%) and increased ($P < 0.01$) the percentage of spermatozoa presenting mechanical acrosome injury (from 6.14 ± 1.93 % to 64.26 ± 9.97%). The percentage of spermatozoa presenting typical acrosome reaction was not significantly different before (3.70 ± 1.22%) and after (0.67 ± 0.67%) centrifugation. The increase of mechanical acrosome damage after centrifugation reinforces the hypothesis that the PVP present in Percoll gradient can be harmful to spermatozoa membranes (4) mainly when Percoll gradients with elevated volumes are used (3). In addition, decapacitating proteins are probably removed during the passage of sperm cells through the colloidal particles present in Percoll medium, a fact contributing to the occurrence of acrosomal damages (1). In conclusion, the Transmission Electron Microscopy distinguished three different types of acrosomal injuries and was effective in characterizing the ultrastructural acrosome damage caused by the Percoll centrifugation. Moreover the mechanical acrosome injury appeared to be the most frequent damage after Percoll gradient centrifugation on cryopreserved bovine spermatozoa.

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Detection of bovine herpesvirus type 5 in frozen spermatozoa following infection of fresh bull semen

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Introduction

Bovine *herpesvirus* type *5* (BoHV-5) is recognized as a neuro-virulent *α-herpesvirus* usually associated with encephalitis in cattle (1). This virus has been isolated occasionally in several countries, but the disease is reported as being prevalent in Brazil and Argentina (2, 3). Natural transmission of BoHV-5 by contaminated semen was recently described in Australia (4) as well as its detection from the semen of a healthy bull (5). Experimental infection of frozen-thawed bull sperm just before in vitro fertilization led to infection of *in vitro*-produced embryos (6). This study was carried out to verify whether BoHV-5 could be found in frozen sperm after an experimental exposure of fresh bull semen to BoHV-5.

Material and Methods

After semen collection and routine evaluations, the ejaculates from three Nellore bulls with proven fertility and no viral contamination were diluted in Tris-citric acid extender containing egg yolk and glycerol. Then, the semen from each ejaculate was splitted into two groups, as follows: I (control) and II (co-incubation with BoHV-5; 10^{-2} $TCID_{50}$/ml; 60 x 10^6 spermatozoa/ml). Afterwards, diluted semen was packed into 0.5 ml straws and cooled to 4°C for 5h. Then, semen samples were exposed to liquid nitrogen (LN) vapor for 20min. before direct immersion in LN. DNA for PCR was obtained from fresh and frozen semen to confirm the absence and presence of viral contamination, respectively. Frozen semen straws from groups I and II were thawed and the spermatozoa were selected by centrifugation on a Percoll gradient. The *in situ* hybridization assay (ISH) was performed to verify BoHV-5 localization.

Results and Discussion

As evidenced by positive fluorescent label after ISH assay, BoHV-5 DNA was found around acrosome-intact spermatozoa at the entire post-acrosomal region and at some parts of the acrosomal cap and tail even after freeze-thawing and centrifugation procedures. These findings are in agreement with other studies with BoHV-1 (8), suggesting that this virus can not only interfere with sperm-zona binding, but also with subsequent fertilization steps. Sperm readily serve as a viral carrier during fertilization (7). In contrast to BoHV-1, BoHV-5 was carried into the ovum during an in vitro fertilization stage without compromising the embryonic development (6). However, in the present study fresh semen was infected with BoHV-5 prior to freezing and, therefore, no information regarding its influence on embryonic development is available. Thus, experiments in progress should demonstrate whether BoHV-5 associated with spermatozoa can interfere with in vitro embryo development.

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Characteristics of frozen-thawed maned wolf (*Chrysocyon brachyurus)* spermatozoa

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Introduction

The maned wolf (*Chrysocyon brachyurus*) is a neotropical canid listed as Near Threatened by the International Union for Conservation of Nature (IUCN; 1). The *ex situ* populations are not viable due to low reproductive efficiency (2), which underline the needs on basic research on reproduction and assisted breeding in captivity to increase the population (3). Thus, the objective of the present work was to freeze the semen of the maned wolf in captivity providing some necessary basis for future studies of its reproductive biology.

Materials and Methods

The study was carried out during the breeding season, in May – June 2010, from one captive maned wolf at the Conservation Breeding Centre of Companhia Brasileira de Metalurgia e Mineração (CBMM), in Araxá, Minas Gerais, Brazil. The wolf was born in captivity, five years old, adult, clinically healthy and had never reproduced before. The semen was collected by digital manipulation and immediately evaluated for volume, pH, vigor, motility, concentration, eosin test (membrane integrity) and morphology. After analysis, the ejaculate was centrifuged at 720 g during 10 minutes. Next, the supernatant was discarded, and the pellet was diluted in Botu-Crio® (Biotech, Botucatu, SP, Brazil) extender, with 7% glycerol, and adjusted to canine semen osmolarity (280 mOsmol), in a sufficient volume to achieve a concentration of $100x10^6$spermatozoa/mL. Sperm samples were loaded in straws (0.25 mL), at room temperature. The straws were put in a refrigerator (4 to 5°C) on a metal platform for 20 minutes, exposed to nitrogen vapor (6 cm from the level of liquid nitrogen) for 20 minutes, and then immersed in liquid nitrogen. After 2 hours of freezing, the samples were thawed at 70°C, during 4 seconds and re-evaluated.

Result and Discussion

Five ejaculates that showed ≥30 x 10^6 spermatozoa were selected for cryopreservation. The mean sperm volume and concentration were 2,4 ml ± 1,24 and 105,65 ± 53,66 x 10^6 cells/ml (n = 5), respectively. The characteristics of the fresh and frozen-thawed semen are shown on Table 1. Although there are studies about the cryopreservation of semen in several species of non-domestic species of canids, this is the first report describing ejaculate characteristics from freshly collected and frozen-thawed maned wolf semen (4-9).

Table 1. Mean results of fresh and frozen-thawed semen from one maned wolf (*Chrysocyon brachyurus*).

Characteristic	Mean Percent (±SEM)	
	Fresh-collected	Frozen-thawed
Vigor (1-5)	3.9 ± 0.55 (n = 5)	2.5 ± 1.54 (n = 5)
Motility (%)	89 ± 5.48 (n = 5)	33.75 ± 25.62 (n = 5)
Life cells in eosin test (%)	82 ± 10.84 (n = 5)	63.60 ± 18.11 (n = 5)
pH	6.8 ± 0.24 (n = 4)	7.04 ± 0.25 (n = 5)
Normal morphology cells (%)	50.75 ± 4.99 (n = 4)	38.88 ± 18.97 (n = 4)
Proximal droplets (%)	10.50 ± 9.96 (n = 4)	4.5 ± 4.14 (n = 4)
Distal droplets (%)	2.88 ± 2.32 (n = 4)	0.63 ± 1.25 (n = 4)
Detached heads (%)	6.25 ± 2.22 (n = 4)	8.75 ± 1.89 (n = 4)
Bent tails (%)	6.13 ± 3.33 (n = 4)	6.63 ± 3.50 (n = 4)
Head abnormalities (%)	10.25 ± 4.73 (n = 4)	10.5 ± 7.82 (n = 4)
Coiled tails (%)	7.25 ± 6.22 (n = 4)	29.25 ± 28.66 (n = 4)
Midpiece abnormalities (%)	5.25 ± 3.71 (n = 4)	2.25 ± 2.96 (n = 4)

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Cryopreservation of epididymal sperm of agouti (*Dasyprocta agouti*) using Tris and powder coconut water (ACP®) extenders

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Introduction

Agouti (*D. agouti*) is a neotropical rodent that inhabits the Northeast of Brazil, and presents potential for economic exploration in captivity based on the production and consumption of their meat for local communities (1). Cryopreservation of epididymal sperm allows the preservation of genetic material even unexpected, and after the animal's death (2).Thus, this research aimed to establish a protocol for the cryopreservation of epididymal sperm of agouti (*Dasyprocta agouti*) using Tris and powder coconut water (ACP®) extenders.

Materials and Methods

Complexes testis-epididymis from 10 healthy males, previously euthanatized were collected. Each epididymis from the same male was dissected and spermatozoa were obtained by retrograde washing using Tris or ACP® extenders. The samples were evaluated for sperm motility and vigor, followed by centrifugation and revaluation of these characteristics. Soon after, epididymal sperm was diluted and frozen in Tris or ACP® plus 20% egg yolk and 6% glycerol and stored in 0.25 plastic straws in liquid nitrogen. After one week, samples were thawed at 37°C/60s, and reevaluated. Differences between treatments were established by Tukey test ($P < 0.05$).

Results and Discussion

After epididymal washing, 77.5 ± 5.0% motile sperm with vigor 3.8 ± 0.2 were obtained in the use of Tris; but 91.5 ± 5.0% motile sperm with vigor 4.7 ± 0.2 were obtained for ACP® ($P<0.05$). After centrifugation, a reduction in the values for sperm motility and vigor was verified for both extenders, being 18.5 ± 5.0% motile sperm with vigor 2.1 ± 0.2 for Tris and motility of 43.5 ± 5.0% with vigor 3.6 ± 0.2 for ACP® ($P < 0.05$). After thawing, sperm motility was better preserved in the use of ACP® that presented values of 26.5 ± 2.6% while only 9.7 ± 2.6% motile sperm were verified in the use of Tris ($P < 0.05$). Otherwise, no statistical difference between extenders was verified regarding sperm vigor with values of 2.6 ± 0.2 for ACP® and 1.2 ± 0.3 for Tris ($P > 0.05$). To the best of our knowledge, this is the first study that presents protocols for epididymal sperm cryopreservation in the agouti. Satisfactory results obtained with the ACP® are attributed to many nutrients such as sugars, glucose, fructose, sucrose and its antioxidant action (3). We conclude that ACP® is more indicated for washing, dilution and cryopreservation of epididymal sperm in agoutis than Tris extender. However, further studies should be conducted in order to improve the efficiency of the protocol for cryopreservation of epididymal spermatozoa in this species.

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Spermatogenic efficiency in four different cricetidae rodents species from the Caraça Natural Reserve

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Introduction

Knowledge on the mammalian reproductive biology and physiology remains extremely limited, especially in relation to the most basic reproductive parameters of wild animals (1). Studies recently developed in our laboratory have shown that, due to several very positive attributes related to testis function, the spermatogenic efficiency in the wild spiny rat (*Trinomys moojeni*), a natural inhabitant from the Caraça Natural Reserve, is by far the greatest among the mammalian species investigated thus far (2). In this context, the main objectives of the present study were to investigate the testis structure, as well as the duration of spermatogenesis and spermatogenic and Sertoli cell efficiencies, in four different wild rodent species belonging to the Cricetidae family and from the same reserve.

Materials and Methods

Six *Akodon cursor*, nine *Akodon montensis*, thirteen *Necromys lasiurus*, and eleven *Oligoryzomys nigripes* were captured in the Caraça Natural Reserve, a fragment of the Atlantic Forest located in the State of Minas Gerais, Brazil. In order to estimate the duration of spermatogenesis, intraperitoneal tritiated thymidine (1μCi/g/BW) injections were administered in two animals of each species. The testes were perfused-fixed in 4% buffered glutaraldehyde and routinely processed for histological and morphometric analyses, as well as for the characterization of the stages of seminiferous epithelium cycle based on the development of the acrosomic system.

Results and Discussion

The data obtained regarding the testis structure showed that the volume density of seminiferous tubules were very high and similar (95-97%) in all four species investigated in the present study. Twelve stages of the seminiferous epithelium cycle were characterized for all species. Based on the relative frequencies of these stages and the analyses of the most advanced germ cell types labeled with tritiated thymidine in the seminiferous epithelium, we observed that these species present a relatively very short duration of spermatogenesis (35-40 days). Regarding the Sertoli cells parameters, the number of these cells found per gram of testis was located on the upper level (~50-70 x 10^6) for the mammalian species already investigated, whereas the values observed for the Sertoli cells efficiency (~8-13) were moderate to high. The combination of the aforementioned parameters resulted in a very high daily sperm production per gram of testis (DSP/g/T), and two ranges of DSP/g/T were observed. In the first range, the two *Akodon* species produced daily 60-65 million of sperm, whereas in the second one *N. lasiurus* and *O. nigripes* produced daily, 75 and 80 million sperm per gram of testis, respectively. Except for the spiny rat (*T. moojeni*), the values obtained for spermatogenic efficiency in the four Cricetidae rodents species investigated in the present study were amongst the highest for mammals investigated up to date. These findings suggest that, probably due to a very short life cycle, these species must reproduce efficiently to ensure their survival. Finally, we expect that the comparative data obtained in the present study will contribute to a better understanding of the reproductive biology of mammals in general and rodents in particular.

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Spermatogonial transplantation in the Nile-tilapia

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Introduction

Germ cell transplantation is a fascinating and powerful technique developed recently, aiming to investigate spermatogenesis and the stem cell biology. This technique also offers great potential for studies involving biotechnology, transgenic animals, and the preservation of the genetic stock of valuable animals or endangered species. Although germ cell transplantation is well characterized for mammals, there is no study utilizing this approach for fish. Tilapias (*Oreochromis niloticus*) are an excellent experimental model due to their fast growth, relative small size when they reach sexual maturity, good adaptability to different environmental conditions and economical importance. Therefore, our main goal in the present study was to investigate the viability of adult tilapias as an experimental model for germ cells transplantation in fishes.

Materials and Methods

The depletion of endogenous spermatogenesis was performed successfully with two busulfan injections [18 mg/kg/BW and 15 mg/kg/BW (two weeks apart)] in tilapias kept at 35ºC. The germ cells to be transplanted were obtained from fifteen sexually mature tilapias that had their testes enzymatically digested with collagenase, trypsin, and DNAse. Spermatogonial germ cells were selected and enriched utilizing Percoll gradient. These cells were labeled with 3.6 X 10–5 M of PKH26 (Red Fluorescent Cell Linker, Sigma) and mixed with a trypan blue solution to check the transplantation efficiency. Eighteen busulfan treated adult tilapias received, into the testis, a total of ~107 donor germ cells. Recipient fishes had their testes analyzed by light and fluorescence microscopy, 15 minutes, 14 hours and 2, 4, 5, 6, 7, and 8 weeks after transplantation, in order to investigate the eventual presence of PKH26 labeled germ cells in the seminiferous tubules

Results and Discussion

Fifteen minutes after transplantation, PKH26 labeled germ cells were observed in the seminiferous tubule lumen of the recipient testes and at fourteen hours, these cells had established contact with the recipient Sertoli cells. At two to six weeks after transplantation, labeled germ cells formed evident spermatogenic cysts showing varied sizes and different stages of development. They were located mainly in the testis regions near to urogenital papillae. The PKH26 labeled cysts surrounded by Sertoli cells were apparently bigger and more frequent at four to six weeks, compared with those observed in tilapias sacrificed two weeks earlier. Finally, seven to eight weeks after transplantation, round and small PKH26 labeled cells were eventually detected into the seminiferous tubule lumem. Moreover, these cells showed scarce cytoplasm (undetectable anti-actin labeling) suggesting that they were released spermatozoa originated from the transplanted spermatogonia.Thus, our results showed for the first time in fish, that spermatogonial germ cells can be successfully transplanted directly into the testes and are capable of colonization, proliferation and differentiation in the recipient testes. In this regard, tilapias can be utilized as an experimental model to investigate the germ cell biology and the testis function in teleosts. Moreover, these findings also provides a new approach for fish bioengineering, preservation of genetic resources of endangered fish species or even fish strains carrying commercially valuable traits.

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Characteristics of fresh maned wolf (*Chrysocyon brachyurus*) semen collected by digital manipulation

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Introduction

The maned wolf (*Chrysocyon brachyurus*) is a neotropical canid that is listed as Near Threatened by the International Union for Conservation of Nature (IUCN). The maned wolf have been housed in zoological institutions from more that 40 years (1), but the *ex situ* populations are not viable due to low reproductive efficiency (2). Thus, the objective of present work was to collect and characterize the semen of captive maned wolf by digital manipulation.

Materials and Methods

The study was carried out from April to June 2010 from two captive maned wolves of unknown fertility at the Conservation Breeding Centre of Companhia Brasileira de Metalurgia e Mineração (CBMM), in Araxá, Minas Gerais, Brasil. The males were restrained physically and 8 ejaculates from wolf 1 (20 Kg, 3 years old) and 11 from wolf 2 (25 Kg, 5 years old) were collected by digital manipulation. Semen volume, motility, vigor, concentration, eosin test and pH were evaluated.

Results and Discussion

Since the first time the two males responded favorably to collection of semen, without agitation or discomfort (Fig. 1). Males remained immobile during the procedure, which lasted from 5 to 10 minutes. The two animals showed rapid response to stimuli on the penis, both wolves underwent mild pelvic movements and wolf 1 vocalized two times while it was ejaculating. From wolf 1 (n = 8) two samples presented sperm cells, while from wolf 2 (n = 11) all the samples collected showed spermatozoa. The results are shown on Table 1. Although conditioning to semen collection by digital manipulation (3) and ejaculatory response to electroejaculation (4) had been described previously for the maned wolf, to date this is the first successful study which presents the fresh semen characteristics and evaluation.

Table 1. Mean results of semen characteristics of two maned wolves.

Semen charactheristics	Wolf 1	Wolf 2
Volume (ml)	1.6 ± 0.37 (n = 8)	2.8 ± 1.7 (n = 11)
Vigor (1-5)	3.5 ± 0.71 (n = 2)	3.5 ± 1.19 (n = 11)
Motility (%)	80 ± 0.00 (n = 2)	74.09 ± 30.73 (n = 11)
Concentration (sptz x 10^6)	20.64 ± 16.07 (n = 2)	95.24 ± 82.68 (n = 11)
Life cells in eosin test (%)	68.50 ± 9.19 (n = 2)	71.50 ± 9.52 (n = 7)
pH	7.48 ± 0.46 (n = 6)	6.8 ± 0.21 (n = 5)

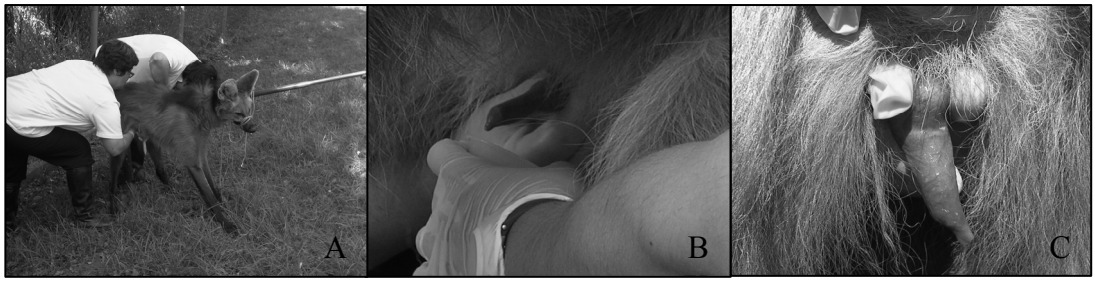

Figure 1. Semen collection in maned wolf. A. Physical restrain. B. and C. Engorged penis during collection.

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Pharmacodynamic of prostaglandin F2α administered through two different routes in cyclic cows

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Introduction

In an attempt to diminish costs, mini-doses of prostaglandin F2α (PGF2α; 5 mg of dinoprost tromethamine) have been administered in intravulvar submucous (IVSM) in estrus synchronization programs, providing satisfactory outcomes (1, 2). In a study of cow genital venous angioarchitecture, it was demonstrated that there are anastomoses between branches of the vaginal vein and veins that drain the cervix, uterine body and horns (3). The aim of this study was to test the hypothesis that PGF2α treatment in IVSM reaches corpus luteum (CL) directly without going through systemic circulation, allowing highest concentrations of non-metabolized prostaglandin in CL.

Materials and Methods

In the first experiment, PGF2α concentration was measured in the uterine vein and jugular vein after administration of 1ml of saline (saline group) or 5mg of dinoprost (PGF2α group). In the second experiment, 13,14-dihydro-15-keto-PGF (PGFM) concentration was determined in the jugular vein after administration of 1ml saline IVSM or different doses of dinoprost intramuscularly (IM; 5 mg or 25 mg) or IVMS (5 mg). In another experiment, pre-synchronized cows received dinoprost on day 5 or 10 after estrus as follow: 25 mg IM; 5 mg IM, 5 mg IVSM and control. Luteolysis was considered when serum progesterone concentrations at day 4 after treatments was lower than 1ng/ml. Serum concentrations of PGF2α, PGFM and progesterone were assessed by specific enzymatic immune assay.

Results and Discussion

In the first experiment, PGF2α at a dose of 5 mg via IVSM resulted in PGF2α levels similar in the uterine and jugular veins (Fig. 1A). This was further confirmed by PGFM levels. The concentrations of PGFM were similar between IVSM and IM after administration of PGF2α (at 5 mg dose) and were lower than the standard dose administrated IM (data not shown). In the last experiment, we compared the effectiveness of 5 mg dose to induce luteolysis in CL in stages of high and low PGF2α responsiveness. Animals that received mini-dose on day 5 did not show functional luteolysis (Fig. 1B). Also, cows that received the mini-dose of PGF2α on day 10 of the estrous cycle presented great variability the luteolysis (3/5 in IM group and 2/5 in IVSM group; Fig. 1C). All the animals received the standard dose presented functional luteolysis. In conclusion, the route (IVSM or IM) of PGF2α administration does not influence serum levels of PGFM and luteolysis. However, reduced doses of PGF2α (5 mg) result in great variability on luteolytic effectiveness.

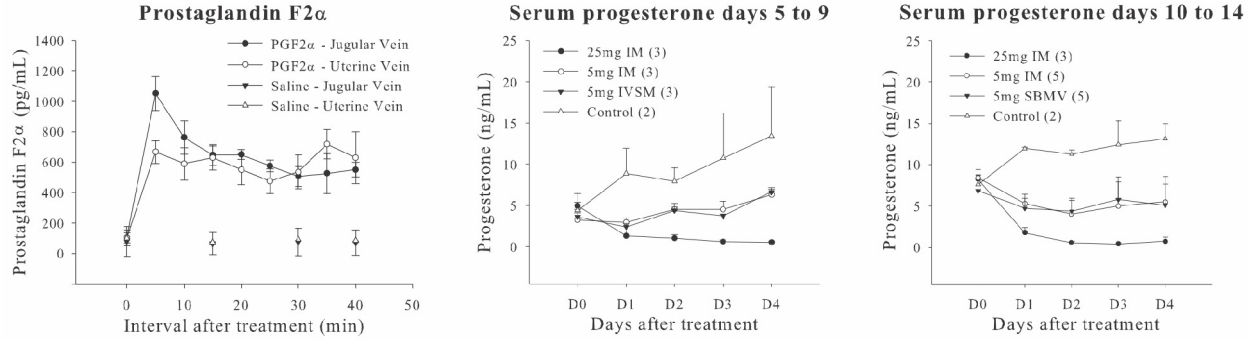

Figure 1. Mean serum PGF2α concentration (pg/ml) in the uterine vein and jugular vein (A). Progesterone concentration (ng/ml) after administration of dinoprost tromethamine on day 5 (B) or day 10 of estrus cycle (C).

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Cloning in goats: comparison of two techniques for oocyte recovery

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Introduction

Animal cloning represents a valuable tool for the production of bioreactor animals (1). However, cloning efficiency in many animal species, such as goats, is directly dependent on the source and availability of good quality oocytes. The aim of this study was to compare the recovery efficiency of goat oocytes using laparoscopic or aspiration of slaughterhouse ovaries.

Material and Methods

For laparoscopic oocyte recovery (LOR), the estrous cycle of 36 goats was synchronized using sponges impregnated with 60 mg medroxyprogesterone acetate (Progespon, Syntex, Buenos Aires, Argentina) inserted intravaginally for 11 days. On day 8, a 50 µg d-cloprostenol (Ciosin, Ford Dodge Saúde Animal, Campinas, Brazil) injection was given intramuscularly (IM), along with the onset of the ovarian stimulation, which was carried out using 120 mg pFSH (Folltropin-V, Vetrepharm, Ontario, Canada) distributed in five IM injections (30/30, 20/20 and 20 mg), 12 h apart. Animals were fasted for 24 h prior to the LOR, which was performed at the time of sponge removal. Conversely, ovaries from 63 well fed goats were collected at a local slaughterhouse and transported to the laboratory in saline at 30^{o}C. All ≥2-mm follicles were counted, followed by follicular aspiration for both groups using a system for small ruminants (WTA, Cravinhos, Brazil). Retrieved *cumulus*-oocyte complexes (COCs) were classified based on morphological criteria (2). Data regarding the mean number of follicles, total COCs, and viable COCs, on a *per* female basis, and the recovery efficiency (COCs *per* number of follicles) were analyzed using the Student´s t-test or the χ^2 test, for $P < 0.05$.

Results and Discussion

After three sections of LOR and five replications with slaughterhouse ovaries, the mean number per goat of aspirated follicles (588/36 *vs*. 1.029/63; 16.3. *vs*. 16.3), total COCs (429/36 *vs*. 885/63; 11.9 *vs*. 14.0) and the number of viable COCs (385/36 and 720/63; 10.7 *vs*. 11.4) did not differ between the oocyte retrieval groups. However, the COC recovery efficiency for slaughterhouse ovaries was higher than for the laparoscopic procedure (86.0% *vs*. 73.0%, respectively). The higher recovery efficiency from slaughterhouse ovaries was likely due to a stricter control over the follicular aspiration process than the remote laparoscopic approach. Visually undetected follicles may also be aspirated when controlled by hand, improving the recovery efficiency. In conclusion, despite the lower COC recovery efficiency, our preliminary results indicate that the *in vivo* laparoscopic procedure was as efficient as the use of slaughterhouse ovaries for the obtention of goat COCs, with both retrieving methods rendering a similar number of good quality oocytes, on a *per* goat basis.

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Comparative study of different cryoprotectants used for slow freezing of cattle *in vitro* produced embryos

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Introduction

It is known that in vitro produced (IVP) embryos are more susceptible to cryoinjury than those in vivo-derived. An efficient cryopreservation protocol for IVP bovine embryos would improve the utilization of this important biotechnology in animal reproduction. This study aimed to evaluate the viability and quality of IVP bovine blastocysts after cryopreservation by slow freezing using 1.5 M ethylene glycol or 1.0 glycerol in association or not with 0.3M sucrose.

Materials and Methods

Oocytes (n = 965) were matured for 24 h in TCM-199 supplemented with 0.2 mM pyruvate, 25 mM sodium bicarbonate and 75 mg/ml gentamicin. Then, oocytes were fertilized with semen of a single Gir bull. Zygotes were cultured in SOFm medium supplemented with 0.5% BSA and 2.5% FCS. Cultures were carried out at 38.5°C in 5% CO_2 in humidified air, in 100 µl droplets of medium covered with mineral oil. On the seventh day of culture, embryos (n = 387) were scored and only excellent to good quality blastocysts were cryopreserved (10 embryos / 0.25 ml straw). The following experimental groups were designed, named according to the cryoprotectant solution used: Glycerol 1.0 M in PBS (G); Glycerol 1.0 M + Sucrose 0.3 M in PBS (GS); Ethylene glycol 1.5 M in PBS (E); and Ethylene glycol 1.5 M + Sucrose 0.3 M in PBS (ES). The embryos were frozen and thawed according to (1). After thawing, embryos were re-cultivated in 100 ml drops of SOFm medium at 38.5ºC and 5% CO_2 in air for 72 h. After 48 h of culture, blastocysts were classified according to their quality (2) and the hatching rate was evaluated at 72 h. The hatched embryos were stained with Hoechst 33342 and the cell number was counted. Data were analyzed by Tukey's test ($P < 0.05$).

Results and Discussion

Results are summarized in Table 1. At 48 h post thawing (hpt), the percentage of grade 1 (G1) blastocysts was higher ($P < 0.05$) in the group ES when compared to G and GS groups, but was similar to E ($P > 0.05$). Embryos cryopreserved with G showed a higher ($P < 0.05$) percentage of G3 embryos when compared to ES, while GS and E groups did not differ ($P > 0.05$) from G or ES. The hatching rate at 72 hpt was similar between groups ($P > 0.05$). Total cell number was higher in E group blastocysts when compared to G group ($P < 0.05$), while GS and ES groups were similar to both ($P > 0.05$). Taken together, data demonstrated that embryo quality, evaluated by embryo score and embryonic cell number, seems to be better in E and ES groups. Since ethylene glycol has the advantage of being less toxic than other cryoprotectants (3) due to its low molecular weight (4), we conclude that cryopreservation of IVP embryos with this cryoprotectant tends to produce better quality embryos after thawing.

Table1. Embryo quality after thawing of IVP embryos cryopreserved with different cryoprotectants.

Treatment	Blastocysts	% Blastocysts (48 hpt)			% Hacthing	Total cell
	(n)	G1	G2	G3	(72 hpt)	number %
G	75	0.0 ± 0.0^{b}	0.0 ± 0.0	100.0 ± 0.0^{a}	19.3 ± 7.1	85.1^{b}
GS	69	0.0 ± 0.0^{b}	20.0 ± 34.6	80.0 ± 24.6^{ab}	44.7 ± 22.6	95.1^{ab}
E	60	4.8 ± 11.7^{ab}	10.6 ± 12.9	69.3 ± 18.4^{ab}	42.2 ± 17.3	108.8^{a}
ES	59	20.1 ± 8.2^{a}	46.7 ± 22.2	48.6 ± 17.2^{b}	46.9 ± 20.0	95.9^{ab}

Results are expressed as mean % ± SD, corresponding to three replicates.
Different letters within the same column indicate differences among treatments ($P < 0.05$).

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Reproductive response of Nelore cows to short flushing

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Introduction

The use of flushing can improve the ovarian follicles growth and the fertility after the estrus synchronization in cattle (1, 2, 3). The objective was to study the efficiency of association of nutritional flushing on the superovulatory response, number, quality and morphology of embryos in Nelore cows.

Material and methods

Nelore cows were submitted to the following treatments: control (TControl, 17 cows) and *nutritional flushing* (T*Flushing*, 17 cows). In T*Flushing* energy supplementation (1% of body weight) was given from 3 days before to 18 days after the beginning of the superovulatory protocol. The superovulation protocol consisted of : Day 0 – insertion of intravaginal progesterone devices plus 2 mg estradiol benzoate (EB) i.m.; Day 7 – beginning of the superovulatory protocol with 140 to 164 mg FSH-p given in decreasing doses; Day 9 – 150 µg $PGF_{2\alpha}$ i.m. in the morning and removal of the progesterone device in the afternoon; Day 10 – 1 mg EB i.m. in the morning; Day 11 – two artificial inseminations were performed after onset of estrus in an interval of 12 hours; Day 18 – embryo collection.

Results and Discussion

The superovulatory response, the total number of structures, the number of viable embryos, nonviables and oocytes were not affected by the energy supplementation. Total structures of about 6.00 ± 1.15 and 3.38 ± 1.19, 3.50 ± 0.82 and 2.08 ± 0.85 of viable embryos, 0.79 ± 0.25 and 0.38 ± 0.26 of nonviable embryos, 0.79 ± 0.22 and 0.38 ± 0.23 of oocytes per cow in T*Control* and T*Flushing* were collected, respectively. A greater presence of morula (38.09%) and of initial blastocyst (25.00%) was found. There were no differences on the quantity of cells (TControl = 56.35; T*Flushing* = 62.69) and of dividing cells characterized by metaphase (TControl= 1.27; T*Flushing* = 1.73) from viable embryos stained with DAPI. Flushing for a short period in Nelore cows did not alter follicular dynamics nor the superovulatory response, in terms of number or viability of the collected structures.

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Kinetic evaluation of goat spermatozoa frozen in media based on powder coconut water (ACP-101®) or TRIS

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Introduction

The coconut water presents important results in goat sperm preservation (1). However, the coconut water has limitations related to storage, availability of fruits and biochemical variations. Thus, powder coconut water (ACP-101®) was developed to overcome these disadvantages (2). The aim of the present study was to evaluate *in vitro* the goat semen frozen in diluents media based on powder coconut water (ACP-101®) and TRIS through kinetic parameters measured by Sperm Class Analyzer® (SCA) computer-assisted system.

Material and Methods

Four adult bucks were used for semen collection by artificial vagina. Each ejaculate was evaluated, divided in two aliquots and diluted in ACP-101® or TRIS. For sperm freezing, the semen was diluted in ACP-101® extender (2,5% egg yolk + 7% glycerol) and TRIS extender (20% egg yolk + 6.8% glycerol). Immediately, the semen samples were kept at 4ºC, glycerolized, placed into straws, exposed to nitrogen vapor (-100ºC) and plunged into liquid nitrogen (-196°C). Straws were thawed after 30 days for both procedures. For motility analysis assisted by SCA each treatment was assessed at 5, 60 and 120 minutes (min) post-thawing. The variables studied were total motility (TM, %), progressive motility (PM, %) and population of rapid spermatozoa (RS, %; Table 1). The data were analyzed using ANOVA and comparisons among the media were determined using Duncans post hoc test ($P < 0.05$).

Results and Discussion

At 5 minutes post-thawing, there were no differences among extenders used for TM and PM variables. Nevertheless, at 5 min, the TRIS extender presented better results when RS was compared to those observed with ACP-101®. At 60 and 120 min post-thawing, the TRIS extender showed increased kinetic compared to ACP-101® to TM, PM and RS. The TRIS extender promoted an increased protection against cryoinjuries, that could be due to the egg yolk quantity in this media (20%), when compared to ACP-101® (2,5%). The sperm cryoprotection of egg-yolk is maintained by the presence of low-density lipoprotein fraction that binds to BSP (Bovine seminal proteins) proteins and this interaction prevents lipid loss from sperm membrane during storage (3). In conclusion, the medium based on TRIS improved *in vitro* viability post-thawing from goat spermatozoa than ACP-101®. In accordance with these results, news studies are necessary to improve the performance of the diluent medium based on ACP-101® for frozen goat semen.

Table 1. Sperm motility analysis at 5, 60 and 120 minutes post-thawing.

	Total motility (%)			Progressive motility (%)			Population of rapid spermatozoa (%)		
Extender	5 min	60 min	120	5 min	60 min	120	5 min	60 min	120 min
ACP-101®	54.5 ± 6.5	17.0 ± 5.4^{B}	1.1 ± 3.7^{B}	27.1 ± 11.3	4.7 ± 5.6^{B}	0.4 ± 1.7^{B}	18.7 ± 11.5^{B}	2.7 ± 4.0^{B}	0.2 ± 0.9^{B}
TRIS	54.6 ±6.3	43.5 ± 9.4^{A}	34.1 ± 8.3^{A}	30.9 ± 12.5	13.9 ± 8.3^{A}	9.7 ± 6.5^{A}	27.5 ± 12.5^{A}	21.1 ± 11.6^{A}	15.4 ± 10.3^{A}

Different letters (A-B) indicate statistically significant difference between extenders ($P < 0.05$).

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Thawing temperature influence on the integrity of membranes in dog cryopreserved spermatozoa

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Introduction

Chilling and freezing of semen as well as artificial insemination (AI) is a biotechnology used a long time in commercial species. However, in dogs, the use of cryopreserved semen in AI is still limited (1). Methods of semen thawing are important for the preservation of sperm viability. Elevation in thawing temperature reduces the occurrence of osmotic damage to sperm cells and prevents ice crystal formation (2). The objective of this work was to evaluate the effect of thawing temperature on the integrity of dog sperm membranes after cryopreservation.

Material e Methods

Dogs of Basset Hound (n = 3) and Rottweiler (n = 3) breeds were used for semen collection (n = 4) by penile manipulation method at three-days intervals. After macro and microscopic evaluations (3), semen samples were diluted in Tris egg-yolk extender (20% egg-yolk; 6% glycerol), packed in straws (0.25 ml), subjected to cryopreservation in automated system and stored in liquid nitrogen (-196ºC). After freezing, semen samples were thawed at 37°C for 1 minute (G1) or 70°C for 6 seconds (G2) and assessed at 0, 30 and 60 minutes after thawing. Semen was evaluated for motility (PM, 0-100%), vigor (0-5), acrosome (iAc; FITC-PNA) and ultrastructural integrity (transmission electron microscopy). Comparisons between groups were performed by ANOVA followed by Tukey test ($P < 0.05$).

Results and Discussion

Results were shown in Table 1. Semen samples from G2 had higher ($P < 0.05$) percentage of cells with PM than the G1 in all incubation times. However, G1 samples had higher ($P < 0.05$) acrosome integrity percentage than those of G2. On ultrastructural evaluation, the G2 had lower preservation of sperm membranes as compared to G1, confirming the results observed in the iAc (FITC-PNA). Based on the results, it can be concluded that thawing at 70°C/6 seconds damages dog sperm membranes. So, it is recommended the temperature of 37°C/60 seconds for thawing dog semen samples.

Table 1. Mean (±sd) of parameters of dog spermatozoa thawed at 37ºC/60 seconds (G1) and 70ºC/6 seconds (G2) after 0, 30 and 60 minutes of incubation (37ºC).

Parameters	Incubation (minutes)					
	0		30		60	
	G1	G2	G1	G2	G1	G2
PM (%)	64.58 ± 13.51^{b}	74.58 ± 8.33^{a}	43.75 ± 16.89^{b}	53.33 ± 13.41^{a}	26.25 ± 17.65^{b}	39.58 ± 16.01^{a}
Vigor (0-5)	3.33 ± 0.48	3.25 ± 0.44	2.46 ± 0.51	2.92 ± 0.28	2.25 ± 0.68	2.58 ± 0.50
iAc (%)	81.83 ± 6.72^{a}	69.88 ± 21.32^{b}	72.79 ± 8.37^{a}	50.46 ± 27.36^{b}	60.63 ± 18.22^{a}	39.54 ± 22.99^{b}

Letters in the same line indicate significant difference ($P < 0.05$) between groups (G1 and G2) at each assessment time (0. 30 and 60 minutes).

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Role of leukemia inhibitory factor associated with the follicle stimulating hormone for in vitro development of sheep isolated preantral follicles

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Introduction

The leukemia inhibitory factor (LIF) is a glycoprotein belonging to the family of interleukin-6 (IL-6). The importance of LIF in folliculogenesis was showed in mice and women, in which it was observed that levels of LIF in follicular fluid increase as the follicle develops, promoting oocyte growth, and maintaining the viability and the development of preantral follicles (1, 2). The aim of this study was to investigate the effects of LIF, with or without follicle stimulating hormone (FSH), on the formation of the antrum and *in vitro* growth of sheep isolated preantral follicles.

Material and Methods

Sheep ovaries were carefully dissected, and the secondary follicles (> 150 mm) were visualized under a stereomicroscope and mechanically isolated with the aid of needles. Growing follicles containing an oocyte surrounded by granulosa cells, with visible and intact basement membrane were selected and cultured individually in drops of 100 µL of medium. The base medium consisted of α-MEM supplemented or not with LIF (10 or 50 ng/ml) in the presence or absence of sequential FSH (day 0-6: 100 ng/ml and 6-12 days: 500 ng/ml). The culture was performed at 39°C and 5% CO_2 for 12 days, and total replacement of the medium was performed on day 6 of culture. A minimum of 35 follicles were used for each treatment. Follicular development was evaluated by the formation of antral cavity and increase of the diameter every six days with the aid of an ocular micrometer. The rate of antrum formation was compared by chi-square, while the follicular diameter was subjected to ANOVA followed by Kruskal-Wallis and Student Newman Keuls test at a significance level of 5%.

Results and Discussion

This study showed that from day 6 of culture all treatments resulted in high rates (above 50%) of antrum formation. On day 12 of culture, the rate of antrum formation in the treatment with 10 ng/ml LIF and FSH was significantly higher (above 90%) when compared with base medium and 50 ng/ml LIF associated with FSH. Moreover, it was observed from day 6 to day 12 of culture a significant increase in the formation of the antrum in all treatments, except the base medium and 50 ng/ml LIF associated with FSH. In relation to follicular diameter, except the base medium (control), all treatments promoted a progressive increase in this parameter until day 12 of culture. Previous studies have reported that the presence of 50 ng/ml LIF in the culture medium increase growth and maturation of the follicles of mouse (1). In addition, this factor was able to promote growth and development of human primordial follicles (2). In conclusion, this study demonstrated that 10 ng/ml LIF with FSH improved the rate of antrum formation of sheep isolated preantral follicles, and that the presence of LIF (10 or 50 ng/ml) with or without FSH increased the follicular diameter after 12 days of culture.

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Sperm parameters and biochemical characteristics of goat seminal plasma during dry and wet season in Northeast Brazil and their influence on cooled semen

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Introduction

Goat fertility is crucial for efficient production under extensive production systems; however, some reports have indicated that climate variations throughout the year induce alterations in goat semen quality. Thus, the present study was conducted to evaluate the effect of season (dry and wet) upon sperm parameters, plasma seminal composition and their influence on cooled semen, at 4°C, of goat raised in Northeast of Brazil.

Material and Methods

Seventeen crossbred adult male goats were used for semen collection by artificial vagina. The ejaculates were evaluated for volume, sperm concentration, sperm vigor, progressive motility and sperm morphology (1). Immediately after collection, semen samples were centrifuged at 550 g for 20 minutes (4°C) and the supernant seminal plasma stored at -18°C until use. The concentrations of citric acid (CA), fructose (Ft), calcium (Ca^{2+}), phosphorus (P), magnesium (Mg) and total protein (TP) were performed using commercial kits. Phospholipase A_2 activity (PLA_2) was quantified according to the technique described by de Haas et al. (2). The semen was diluted in coconut water solution, citrate and egg yolk diluents and cooled at 4ºC during 2 hours and after 2, 24 and 48h sperm vigor, morphology, percentage of motile cells and degradation motility rate were determined. The thermoresistance test was also performed (3). The values were expressed as mean ± SEM and the comparisons were determined using Tukey's test at $P < 0.05$.

Results and Discussion

We observed that P, Mg, CA, Ft e TP concentration were significantly lower during the dry season. These results probably be due to higher temperatures, which promove heat stress in the bucks. Moreover, semen cooled at 4ºC during 48 hours show decreased total motility, vigor and sperm morphology during the dry season as compared to wet season. The motility degradation rate (DMR) after 2 hours of cooling was higher in dry season (Table 1). This fact could be attributed to PLA_2 that also increased in this season. This enzyme hydrolyzes phospholipids from egg-yolk into unsaturated fatty acids and lysophospholipids, which are toxic to spermatozoa (4, 5). Based on these findings, it was concluded that the best period of the year for caprine semen cooling is the wet season.

Table 1. Sperm parameters (Mean ± S.E.M.) of goat semen samples during dry and raining season and subjected to cooling at 4ºC during 48 hours.

	Total motility (%)		Vigor (0-5)		DMR* (%)		Morphology normal sperm	
Hours	Dry	Rainy season	Dry	Rainy season	Dry	Rainy season	Dry	Rainy season
02	51.0 ± 4.2^{aB}	58.2 ± 2.6^{aA}	2.1 ± 0.2^{aB}	2.6 ± 0.1^{aA}	$57.6 \pm 9{,}1^{aA}$	28.2 ± 6.2^{aB}	80.8 ± 1.9^{aB}	84.9 ± 2.0^{aA}
24	40.1 ± 6.4^{bB}	51.7 ± 3.5^{bA}	1.6 ± 0.3^{bA}	2.3 ± 0.2^{bB}	50.5 ± 9.1^{aB}	28.2 ± 7.3^{aB}	81.8 ± 2.0^{aB}	85.9 ± 2.0^{aA}
48	30.7 ± 6.8^{cB}	44.2 ± 4.1^{cA}	1.3 ± 0.3^{cB}	2.1 ± 0.2^{cA}	43.5 ± 9.0^{bB}	27.1 ± 7.4^{aB}	81.9 ± 1.7^{aB}	86 ± 1.7^{aA}

Values with a and b indicate comparison among times in the same column, and A and B indicate comparison between seasons in the same row ($p < 0.05$) and ($p < 0{,}0001^{*}$).

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In vitro meiotic delay of ovine oocytes with cycloheximide

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Introduction

The **in vitro** production of ovine embryos (IVP) is a promising biotechnology which allows the recovery of cumulus-oocyte complexes (COCs) from follicles at different stages of development and the use the same oocytes donor from pre-puberty to senility and the *post-mortem* period, providing better production efficiency of the herd sheep (1, 2). However, despite of recent scientific advances, there are some obstacles related to the process of *in vitro* oocyte maturation limiting the success of biotechnology in the sheep rearing. The simple removal of the COCs from the follicular environment promotes the spontaneous meiotic resumption independently of some molecular and structural changes that characterize the cytoplasmic maturation and are essential to support the subsequent fertilization and embryo development (1). A strategy to improve the *in vitro* oocyte developmental potential is temporarily to block the nuclear maturation with the use of pharmacological meiotic inhibitors (3).

Materials and Methods

This experiment aimed to evaluate the effectiveness of cycloheximide (an inhibitor of protein synthesis), at concentration of 1 µg/ml, to inhibit oocyte meiosis**.** This concentration was based on previous tests and literature information (4).For that, sheep ovaries obtained from slaughterhouses were transported to the Laboratory of *In Vitro* Embryo Production of the Department of Animal Reproduction and Veterinary Radiology at Veterinary Hospital - UNESP - Botucatu – SP, in thermos containing saline solution at 37°C. After aspiration with syringe needle attached, COCs were classified as grade 1 and 2 (5) and were submitted to culture in maturation medium TCM 199 (Tissue Culture Medium-199) supplemented with fetal bovine serum, cysteamine, pyruvate, penicillin, LH (Luteinizing Hormone) and FSH (Follicle-Stimulating Hormone) with or without (control group) 10 µg/ml of cycloheximide, for 24 hours in an incubator at 38.5°C with 5% of CO2 in air. To evaluate the nuclear maturation, the denuded oocytes stained with Hoechst 33342 were analyzed in a fluorescence microscope. The experimental design was completely randomized with two treatments (control group and group treated with cycloheximide) and 6 replicates with a total of 218 oocytes evaluated (92 oocytes in the control group and 126 oocytes in the treated group). In this experiment, about 800 ovaries were needed to obtain 218 oocytes grade 1 and 2.

Results and Discussion

The results demonstrated that the cycloheximide at a concentration of 1µg/ml was effective in delaying the progression of nuclear maturation in 90% of oocytes, being 6% of oocytes maintained at germinal vesicle breakdown and 84% in metaphase I. None of the oocytes (0%), prematured with cyclohexemide reached the metaphase II stage, whereas in the control group, 81% of oocytes were at metaphase II, 13% were at metaphase I and 3% were at germinal vesicle breakdown, after 24 hours of maturation. The percentage of degenerated oocytes in the presence of the inhibitor was slightly higher than in the control group (10 and 3% respectively). Therefore, it seems that cyclohexemide is efficient in delay the progression of nuclear maturation in ovine when used at an appropriate concentration (1 µg/ml), and can be used in the process of *in vitro* production of embryos for the purpose of establishing the synchrony between nuclear and cytoplasmic maturation.

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Structural characterization of lipids in horse embryo obtained by mass spectrometry

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Introduction

Matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) allows not only the fast and efficient detection of various lipids classes, but also the study of their structure by fragmentation (MS/MS) experiments (1). The aim of this study was to characterize the lipid classes present in one single equine embryo by MALDI-MS, and to study some lipid structures by collision induced dissociation (CID) experiments.

Material and Methods

One equine embryo recovered by uterine wash on day 8 (D8) post ovulation was placed in 50/50 (v/v) methanol/phosphate buffer solution and transported at 4ºC to the laboratory. MALDI-MS spectra were acquired in the positive (using 2,5-dihydroxybenzoic acid as MALDI matrix) and negative (using -aminoacridine hemihydrate as MALDI matrix) ion modes in a MALDI Synapt HDMS mass spectrometer (Waters, Manchester, UK) at the *m/z* 400-1000 range. Spectra processing was performed using the MassLynx 4.0 software (Waters, Manchester, UK). Besides obtaining the lipid profile at both ion modes, some ions were isolated in the positive and in the negative ion mode for MS/MS experiments.

Results and Discussion

In the lipid profile in the positive ion mode, mainly phosphatidylcholines (PC), phosphatidylethanolamines (PE), sphingomielyns (SM), and triacylglyceols (TAG) species were observed, while in the negative ion mode, PE and phospathidylinositols (PI) were detected. MS/MS spectrum in the positive mode of *m/z* 760.6 (attributed as PC34:1) depicted characteristic PC fragments of *m/z* 184.1 (choline polar head), and the neutral loss (NL) of 183 (phosphorylcholine). For the ion of *m/z* 766.6 (attributed as PE 38:5), we observe the NL of 140, characteristic of PE. For the ion of *m/z* 808.7 (attributed as PC 38:5), besides the fragment at m/z 184.1 at the NL of 183, it was possible to observe the loss of trimethylamine (ion at *m/z* 749.6), and the cyclophosphane (ion at *m/z* 147.0). Finally, for the negative ion mode, we isolated and fragmented the ion at *m/z* 863.6, which was attributed as PI 36:1 due to the presence of various characteristic ions reported for this lipid species: At *m/z* 153 (glycerol phosphate -H_2O-H), 223 (phospho inositol-2H_2O-H), 241 (phospho inositol-H_2O-H), 281 (oleic acid), and 581.3 (lysophosphoinositol-H_2O-H). The MALDI-MS allows the detection of PC, SM, PE, PI and TAG lipid species, as well as fast and confident characterization of its lipid structure from one single equine embryo. These results may contribute to a better knowledge of equine embryo metabolism and to cryopreservation studies.

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Effect of addition of caffeine and reduction of the volume of medium for *in vitro* fertilization on the rate of sperm penetration in cattle oocytes

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Introduction

Since the beginning, in vitro fertilization (IVF) has been used to study the maturation, fertilization and embryo development. Ideally, IVF should provide high rates of fertilization. For this purpose, several compounds have been used to promote and optimize sperm capacitation (1, 2). Caffeine is a phosphodiesterase inhibitor, an enzyme that increases the concentration of cyclic AMP during sperm capacitation, modulating the acrosome reaction and enhancing sperm penetration (3).

Material and Methods

A group of 240 bovine oocytes obtained from ovaries of slaughterhouses in Campos dos Goytacazes, RJ, Brazil, were matured and fertilized (1.6 x 10^6 sperm/mL) in vitro in four experimental groups (CONTROL, G1, G2 and G3**)** with 20 oocytes per drop covered with mineral oil. All the experiments were repeated 3 times. Oocytes in the control group were fertilized in 100 µL (1 oocytes:5 µL) of fertilization medium talp-fert (IVF medium), in group 1 (G1) 100 µl of IVF medium supplemented with 2M caffeine; (1 oocyte:5 µL) were used; in group 2 (G2) there was a reduction of medium volume (from 100 µL to 40 µL, 1 oocyte: 2 µL), and oocytes were fertilized in 40µl (1 oocyte: 2 µL) of IVF medium without caffeine, and in group 3 (G3) we evaluated the reduction of fertilization medium (40 µL, 1 oocyte: 2 µL), and the addition of 2M caffeine. After fertilization (18 hours), 125 presumptive zygotes chosen randomly from each treatment were fixed in methanol/acetic acid overnight. Samples were stained with 2% acetic orcein and evaluated by light microscopy and classified as penetrated or not penetrated (the zygotes that had only structures related to the genetic material of females), zygotes with two pronuclei (male and female) or with the presence of de-condensed sperm head.

Results and Discussion

The statistical analysis was performed by confidence interval proportion test ($P < 0.05$) and there was no significant difference in the fertilization rates among groups C (75.36% ± 0.4), G1 (65.62% ± 0.4), G2 (84.61% ± 0.3) and G3 (74% ± 0.4). As a conclusion, the addition of 2M caffeine in fertilization medium and the reduction of drop volume did not affect fertilization rates of bovine oocytes.

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Cryopreservation of stallion epididymal sperm using different freezing rates: preliminary results

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Introduction

Unexpected death, catastrophic injury, castration or any other event that makes both semen collection and mating impossible can be costly in terms of the potential loss of genetically valuable stallion genes. There are limited data regarding survival and fertility of frozen-thawed epididymal stallion sperm, although the first pregnancy using frozen-thawed stallion spermatozoa was reported in a mare inseminated with epididymal spermatozoa. The aim of the present study was to evaluate the influence of three different freezing rates on sperm viability of samples obtained from stallion epidydimal cauda.

Material and Methods

Six Brazilian Jumping horses, aged 2.5 years old were castrated and sperm were immediately harvested from the epididymis. Spermatozoa from the cauda were obtained using retrograde flushing with Botu-SemenTM extender. The samples were incubated at room temperature (25ºC) for 15 minutes, and then centrifuged at 2200 rpm for 10 minutes. The supernatant was discharged and the pellet resuspended using the Botu-Crio™ extender. Before freezing, the semen was packed into 0.5 ml straws and maintained at 5ºC for 20 minutes. The samples were frozen using three different freezing rates: A) -15oC/min in the isothermic box (20 minutes at 6 cm above liquid nitrogen); B) -40oC/min (TK 4000 machine) and C) -50oC/min (Mini-digiticool 1400 machine). The straws were thawed at 46ºC for 20 seconds and evaluated by CASA (HTM IVOS 12). Plasma membrane integrity was evaluated using fluorescent probes (CFDA and PI). Data were analyzed by ANOVA, followed by Tukey test.

Results and Discussion

The results were described in the Table 1.

Table 1. Mean (± SD) values for Total Motility (TM), Progressive Motility (PM), and Plasma Membrane Integrity (PMI) of frozen-thawed semen samples from equine epididymal cauda using 3 different methods of freezing: isotherm boxes (BOX), TK 4000 machine (MAC) and Mini-digiticool 1400 machine (BIO).

	BOX	MAC	BIO
TM	34.6 ± 23.54	34.6 ± 21.15	33.2 ± 22.79
PM	17 ± 14.56	16 ± 10.84	18 ± 13.25
PMI	47 ± 10.59	45 ± 15.03	51 ± 16.33

Preliminary results indicate that there was no difference on sperm parameters among the used freezing rates. However, it may be expected some difference among freezing rates when using a higher number of stallions. Results presented in this study suggest that cauda epididymis stallion sperm may be frozen using different freezing rates. These provide a last option for the owner to store genetic material of a high valuable stallion in case of death or permanent injuries. More studies are necessary to optimize the use of frozen epididymal semen in commercial programs and associated to other technologies such as the Intracitoplasmatic Sperm injection (ICSI).

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High mitochondrial potential and mtDNA copy number associated with DNA fragmentation in bovine oocytes treated with Butyrolactone I

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Introduction

Oogenesis can be extended in vitro by blocking meiotic resumption with the kinase inhibitor butyrolactone I (Bl-I) to premature oocytes before final in vitro maturation. Apart from their role in providing energy resources for early development, mitochondria are directly involved in cell death pathways. The present study aimed to assess the effects of Bl-I during a prematuration culture of bovine oocytes on nuclear and cytoplasmic maturation, measured by analysis of mitochondrial membrane potential, copy number of mitochondrial DNA (mtDNA) and DNA fragmentation.

Material and Methods

Oocytes were prematured (pre-IVM) during 24h with 100 µM Bl-I diluted in TCM199 supplemented with 0.2 mM pyruvate. After pre-IVM, oocytes were matured (IVM) in modified SOF medium (mSOF; 108 mM NaCl, 7.2 mM KCl, 0.5 mM $MgCl_2$, 2.5 mM $NaHCO_3$, 1.7 mM $CaCl_2$-H_2O, 0.5 mM glucose, 0.33 mM sodium pyruvate, 3 mM sodium lactate, 2 mg/ml BSA, 150 mg/ml gentamicin, 0.01% phenol red, 1.4 mM glycine, 0,4 mM alanine, 1 mM glutamine, 1% nonessential amino acids and 2% essential amino acids) supplemented with 0.8% BSA and hormones during 24 h (control group: C 24 h) or during 20h (Bl-I group: oocytes pre-IVM 24h + MIV 20 h). Cultures were carried out at 38.5°C in 5% CO_2 in humidified air, in 100 µl droplets of medium. A group of oocytes was also analysed immediately after aspiration from the follicle (control 0 h). After pre-IVM and IVM, oocytes were stained with 500 nM mitotracker red to assess the mitochondrial membrane potential ($\Delta\Psi m$) and with 10 µg/ml Hoescht 33342 to evaluate the nuclear maturation (n = 176). Images were captured by Olympus confocal microscope and analysed with Fluoview software. mtDNA analysis of individual oocytes (n = 33) were performed according to (1) and only oocytes without polar body (PB) after pre-IVM were selected for mtDNA analysis. The TUNEL assay was used to detect oocyte DNA fragmentation associated with the later stages of apoptotic cascade (n = 302). Comparisons of means were evaluated by ANOVA and Tukey's test ($P < 0.05$).

Results and Discussion

After pre-IVM, it was observed that 60.7% (Bl-I) of oocytes were in germinal vesicle stage (GV - immature; $P < 0.05$), indicating that Bl-I blockage was only partial. Nonetheless, after IVM, 79.1% (C 24h) and 79.8% (Bl-I; $P > 0.05$) oocytes reached metaphase II (MII), indicating that in vitro meiotic resumption was normal. The $\Delta\Psi m$ was higher in oocytes recently aspirated from the follicle (C 0 h) than in those pre-IVM with Bl-I (1274.5 ± 121.4 and 826.1 ± 59.6 units of fluorescence intensity, respectively; $P < 0.05$), indicating a loss of mitochondrial activity. However, after IVM, the fluorescence intensity was higher in Bl-I than in controls (2754.3 ± 57.1 vs. 881.4 ± 5.0; $P < 0.05$), indicating an abnormal increase in mitochondrial activity. Regarding the mtDNA, we found 34.1×10^4 (C 0h) and 52.5×10^4 copy numbers (Bl-I; $P < 0.05$) per oocyte after pre-IVM, and 39.7×10^4 (C 24 h) and 49.5×10^4 (Bl-I; $P > 0.05$) after IVM, indicating that mtDNA numbers increase in oocytes that underwent pre-IVM with BI-1. Although there was no significant increase in the percentage of TUNEL-positive oocytes after pre-IVM (0% - C 0 h and 24.1% - Bl-I; $P > 0.05$), the pre-IVM + IVM culture resulted in higher percentage of TUNEL-positive oocytes (10.5% - C 24 h and 25.6% - Bl-I; $P < 0.05$), indicating an increase in apoptosis by Bl-I blockage. Together, these results suggest that Bl-I blockage of bovine oocytes leads to an abnormal increase in mtDNA numbers after pre-IVM as well as in mitochondrial activity after IVM that is associated with the fragmentation of the meiotic chromatin.

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In vitro production of embryos in sheep: a report of pregnancy success after long periods of oocytes and embryos transport

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Introduction

The purpose of this study was to report the feasibility of transporting sheep oocytes and embryos produced in vitro over long distances.

Material and Methods

Multiparous Santa Ines ewes (n = 5) with an average body condition score of 3 were submitted to the hormone treatment named "One Shot"(1). This treatment consisted of a subcutaneous insertion of a device containing 0,3 mg of progesterone (CIDR®, Pfizer) on D0, which was kept for 11 days. Thirty six hours prior to the progestagen withdrawal (D11), 0.04 mg sodic D-cloprostenol (Preloban®, Intervet), 200 IU of FSH (Pluset®, Hertape Calier) and 300 IU of eCG (Folligon®, Intervet) were administered. After the progestagen withdrawal oocytes were retrieved via laparotomy. Cumulus oocyte complexes (COC) classification was performed under stereomicroscope according to Seneda (2). The selected COC were placed in TCM 199 culture medium, aerated with 5% CO_2, and stored in a transport incubator at 38.5°C. Oocytes were transported for 8 h by car (583 km) to the laboratory for *in vitro* embryo production (IVP). After a maturation period of 22 hours, oocytes were fertilized with ram semen. Zygotes were cultured in G1/G2 culture medium (3) for 84 hours. Embryos were shipped back to the original farm by the same transport conditions described above. Viable embryos were transferred to recipients 4 days after oocyte retrieval. Recipient ewes (n = 7) were prepared previously with 1,5 mg norgestomet on the same day that oocyte donors received the progesterone device (D0). Upon progestagen device withdrawal, on D9, recipients received 400 IU of eCG and 0,04 mg of sodic D-cloprostenol. On D16, recipients that had at least one corpus luteum (CL) received 3-4 embryos in the morula stage by laparotomy. Embryos were transferred in the cranial portion of the uterine horn ipsilateral to the CL. Pregnancy diagnosis was performed by transrectal ultrasonography 30 days after embryo transfer.

Results and Discussion

Each donor produced 9.8 ± 5.7 oocytes (49/5), of which 4.5 ± 2.9 (23/5) were grade I, 1.6 ± 0.9 (8/5) grade II, 1.8 ± 0.8 (9/5) grade III, 1.0 ± 1.7 (5/5) were naked oocytes and 0.8 ± 0.8 (4/5) were degenerated. All oocytes were sent to the laboratory for fertilization and culture. The embryo production per donor resulted in 9.8 ± 3 (49/5) embryos, and 5.4 ± 3.1 (27/5) of them were grade I morulae; 2.4 ± 1.67 (12/5) grade II morulae and 2 ± 2 (10/5) were degenerated embryos. Twenty five grade I morulae were transferred into 7 recipients. One pregnancy (14.3%; 1/7) was confirmed in a recipient that had 4 CLs. One healthy lamb was born after an eutocic parturition. The low freezing efficiency sheep embryos produced *in vitro* limits the use of this biotechnology. The embryo culture after long distance transport is an alternative to minimize this problem. These results indicate the possibility of sheep IVP after transport of oocytes and embryos for long distances, as recently demonstrated by Pontes in cows (4). The improvement of this technique can open a possible alternative for the multiplication of sheep genetics.

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Influence of a previously refrigerated container on the transport of equine semen

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Introduction

Transport of cooled semen has been routinely used in equine breeding industry (1). Most breed registries within Brazil now authorize registration of foals conceived through the use of transported-cooled semen. However, inadequate transportation can result in loss of fertilizing capacity of equine sperm (2). In many stud farms in Brazil, semen samples are transported by outsourced services. Therefore, transportation conditions lack adequate control. For example, semen is frequently packed in a previously refrigerated container, which can lead to a cold-shock on spermatozoa. The current study aims to compare the effect of storage of equine semen in containers refrigerated previously or not on sperm viability.

Materials and Methods

For this experiment, 14 Quarter Horses stallions with known fertility were used. One ejaculate was obtained from each stallion and then diluted in a skim-milk based extender (Botu-Semen^{TM}) in a concentration of $50x10^6$ sperm/ml. Aliquots of 7mL of extended semen from each stallion, totalizing 98 mL per container, were stored in containers developed to achieve 5^o or 15^{o}C, either refrigerated previously or not. The samples were analyzed after 12 and 24 hours of storage. Sperm variables were evaluated by CASA (HTM-IVOS 12, Hamilton Thorne Research, USA). In addition, plasma membrane integrity was evaluated by fluorescent probes carboxyfluorescein diacetate and propidium iodide. All variables were analyzed by unpaired *t* test using GraphPad InStat Version 3.06, considering significant effects in which $P < 0.05$.

Results and Discussion

There were no significant differences ($P > 0.05$) on total motility (TM), progressive motility (PM) and percentage of rapid sperm (Rap) on previously refrigerated or non-refrigerated containers, either at 5 or 15^{o}C (Table 1). It has been shown that cold-shock occurs in samples stored in pre-refrigerated containers, as demonstrated by significantly lower values on plasma membrane integrity ($P < 0.05$). Cold-shock mechanisms are not well understood but membrane status is impaired when temperature is decreased because of the loss of phospholipids (2). According to Mooran et al. (3), sperm which are exposed to a rapid decrease in temperature undergo cold-shock damage characterized by abnormal motility patterns, rapid loss of motility, and membrane damage. Based on the results of the present experiment we can conclude that the inadequate refrigeration on semen transport decrease sperm viability, and can result in loss of fertilizing capacity of cooled equine semen.

Table 1. Sperm parameters of samples packed in pre-refrigerated (PR) or non pre-refrigerated (NPR) containers submitted to refrigeration for 12 and 24h at 5 or 15^{o}C.

Sperm	5^{o}C/12 h		15^{o}C/12 h		5^{o}C/24 h		15^{o}C/24 h	
parameters	NPR	PR	NPR	PR	NPR	PR	NPR	PR
TM (%)	70.9	65.9	68.1	64.6	61.6	53.9	60.3	55.6
PM (%)	34.5	31.1	39.9	34.6	27.4	21.6	31.0	27.1
Rap (%)	60.9	58.1	58.9	55.9	52.9	47.4	49.1	47.6
PMI (%)	61.6	53.8*	59.1	50.2*	49.1	42.2*	53.1	42.2*

PMI= Plasma Membrane Integrity. *Indicates differences within the same row in each treatment ($P < 0.05$).

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Isolation, culture and cryopreservation of bovine amniotic cells with Dimethyl sulfoxide, Dimethylformamide or Glycerol

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Introduction

Adult somatic cells can undergo nuclear reprogramming after fusion with a mature oocyte, a process known as somatic cell nuclear transfer or cloning. An important requisite is the availability of cells able of undergoing reprogramming to support embryonic and fetal development after nuclear transfer (1). In this context, the bovine amniotic cells, which are relatively less differentiated, have a potential for use in nuclear transfer. Thus, this study seeking alternatives to improve the efficiency of bovine nuclear transfer aimed to evaluate the possibility of isolation and storage of amniotic cells, comparing the efficiency of three cryoprotectant solutions.

Materials and Methods

Amniotic liquid was collected from four bovine placentae ranging from 70 to 90 days of gestation. The amniotic sac was exposed and the collection of 15 mL was done with needle and syringe to isolate amniotic cells. The amniotic liquid was centrifuged for 5 minutes at 200g. Then, the cellular sediment was diluted in Amniomax medium and cultured in incubator at 5% CO_2 and 38,5°C until the cells reached confluency. Next, cells were resuspended and distributed in 3 cryopreservation solutions containing 10% Dimethyl sulfoxide (DMSO), 5% Dimethylformamide (DMF) or 7% Glycerol. Cells (2 x 10^6 cells/mL) were loaded into 0,25 mL straws and were frozen at -80°C for 24 hours and then stored in liquid nitrogen. The straws were thawed at 37°C/30s. The viability rate of cells was evaluated with *trypan blue* stain (TB). The thawed cells were re-cultivated in Amniomax medium and time to confluence was to analyzed.

Results and discussion

The cryoprotector medium with DMSO preserved 84.50 ± 9.53% of amniotic cells with intact membrane, which was superior to cryoprotector medium containing DMF, that preserved 42,00 ± 13,92% of the same cellular type (Fig 1). The cryoprotector medium supplemented with Glycerol preserved 63.00 ± 18.18% of cells and did not differ from the others treatments (P > 0.05; Fig. 1).

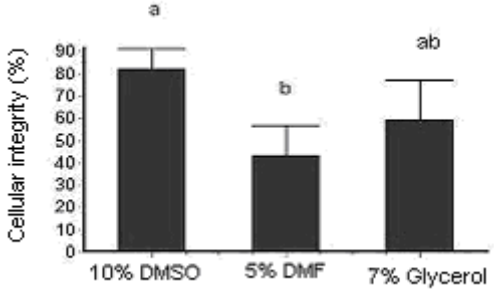

Figure 1. Integrity of the cryopreserved amniotic cells in protection solutions containing DMSO, DMF or Glycerol diluted in Amniomax medium.

After the celular re-culture, only cells frozen previously on DMSO cryprotection reached confluence in 6 days. This study demonstrated to be possibility to isolate and cryopreserve bovine amniotic cells for use in the nuclear transfer technique. The cryoprotection medium most appropriate was DMSO.

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Procedure for the reconstruction of inner cell mass and trophoblast in bovine embryos

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Introduction

The efficiency of embryonic chimerism tends to decrease when embryos in advanced stages of development, such as morulae and blastocysts, are used. To perform the inner cell mass (ICM) transfer to a trophectoderm (TE) receptor it is essential to use embryos at an advanced stage and that contain a blastocoel. This method of embryo reconstruction has been performed only by the micromanipulator microinjection method. This study aimed to validate a manual procedure to reconstruct embryos using the method of ICM and trophoblast approximation in the presence of an agglutinating agent.

Material and Methods

Zebu embryos produced in vitro were used 8.5 days after fertilization. To conduct the manual bisection of ICM and trophoblast, hatching or hatched blastocysts were placed into 3 µl-microdrops of protein-free HSOF medium. The bisection was executed with a microblade (Ultra-Sharp Splitting Blade, Bioniche, USA) under stereomicroscope (35X magnification). Twenty-four half-structures were joined (ICM-trofoblast) and transferred to an embryo reconstruction plate, where they were keept for 3 min in drops containing 500µg/mL phytohemagglutinin-L, before the approximated pairs be transferred to SOF medium in cell aggregation WOW micro-wells (5) to *in vitro* culture (38.5°C; 5%O_2 and 5% CO_2). The agregation of structures was evaluated after 24 h, while the reexpansion of blastocel was evaluated after 48 h.

Results and Discussion

The embryo immobilization for hand-bisection (no micromanipulator) was difficult, because the embyos used were not a compact cell mass like the 8-cell embryo, morulae; or an almost compact structure like the early blastocyst. The embryo changed position frequently and turned around its axis when the blade was pressed to cut and separate the structures. To facilitate the bisection, microdrops were made using a medium free of protein or other compounds that could prevent adherence of the embryo to the plate. Then, the structures were released from the plate by adding to the drop 1 µl of bovine fetal serum and pipeting. Three of the twelve recultivated structures were aggregated after 24 hours of culture and re-expanded after 48h.The method of ICM isolation by manual-bisection with microblade was efficient, but it requires training and skills from the executor. The technique of ICM aggregation with trophoblast by approximation in the presence of an agglutinating agent can be considered efficient regarding the execution, the structural reconstruction of the blastocyst and the reexpansion of the blastocel.

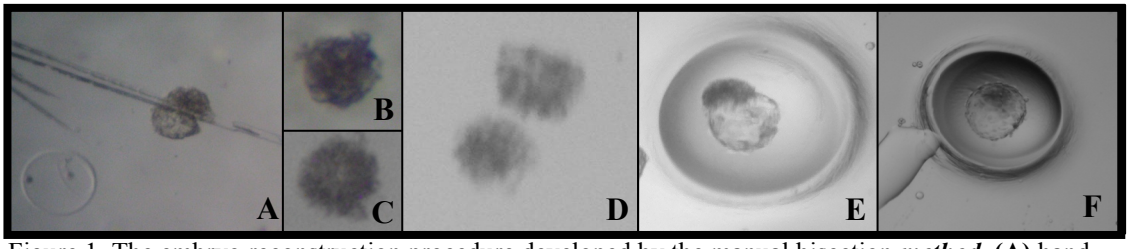

Figure 1. The embryo reconstruction procedure developed by the manual bisection ***method*. (A)** hand-**bisection** of the ICM and trophoblats. Isolation of ICM (B) and trophoblast (C). (D) Embryo reconstruction. Chimeric blastocyst 24 hours (E) and 48 hours (F) after aggregation.

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Morphologic evaluation of apoptotic and viable cells of ovine embryos stored frozen or vitrified with dimethylformamide and ethylene glycol

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Introduction

Ovine pregnancy rates after transfer of cryopreserved embryos are still inconsistent. The aim of the present work was to evaluate the viability of Santa Inês breed embryos cryopreseved by the conventional method and vitrification. In the vitrification medium two cryoprotective agents were tested: dimethylformamide (DMF), which has not been used yet to cryopreserve ovine embryos, and ethylene glycol (EG), which is frequently used in mammalian embryo cryopreservation.

Material and Methods

Ovine embryos obtained *in vivo* were submitted to three cryopreservation treatments: T1) Conventional freezing with 1,5M EG (n = 37), T2) Vitrification with EG (n = 34), and T3) Vitrification with DMF (n = 33). In the conventional method, the embryos were loaded into 0.25 ml straws, while in vitrification they were loaded in "Open Pulled Straw" (OPS). The embryos were thawed at 35ºC for 30 seconds for the conventional method and warmed at room temperature in air for 3 seconds after vitrification. All embryos were rehydrated in sucrose solutions and cultured at 38.5°C, 5% of CO_2 in air and saturated humidity for 48 hours. The embryos were evaluated for re-expansion and hatching rate, viability with Hoechst 33342/ Propidium iodide (PI), apoptotic rate by TUNEL procedure (Promega Kit, USA), and morphology, after inclusion in glycolmethacrilate resin (Historesin Leica, USA), which had not been used yet for embryonic evaluations.

Results and Discussion

The re-expansion rate of T1, T2 and T3 was of 54% (20/37), 47% (16/34), 36.6% (12/33), respectively. The hatching rate of T3 was significantly lower than T1 and T2 ($P < 0.05$). The vitrified embryos (T2 and T3) showed lower rate of viable cells than the control and conventional freezing method, while the cellular apoptotic rates were higher in the EG treatments (T1 and T2; Table1). The major damages observed with the inclusion in glycolmethacrilate resin were in the DMF treated embryos (T3). Because no apoptotic cells were detected in these embryos, it is possible that DMF induced a very high and acute toxicity causing death by necrosis. This could explain the high grade of plasma membrane rupture and cytoplasmic fragmentation observed by light microscopy in this group. The DMF was not efficient to vitrify ovine embryos. In contrast, the conventional freezing method with EG was feasible for ovine embryo cryopreservation.

Table 1**.** Hatching, viability (Hoechst 33342/PI) and apoptosis rate (TUNEL) of conventional frozen and vitrified ovine embryos with ethylene glycol (EG) and dimethylformamide (DMF).

Treatments	% Hatched embryos (n)	% Viable cells (n)	% Apoptotic cells (n)
Conv. Freezing (T1)	40.5 (15/37)[a]	76.7% (33.4/43.5)[a]	14% (12.1/98.2)[a,b]
Vitrification EG (T2)	35.3 (12/34)[a]	33.6% (21.6/64.2)[b]	30% (15.7/72.6)[b]
Vitrification DMF (T3)	15.5 (5/33)[b]	8.1% (5.6/68.8)[b]	0% (0/56)[a]
Fresh (control)	-	98.2% (84.1/85,6)[a]	8% (6.0/68.1)[a,b]

[a, b]Values in the same column with different superscripts differ significantly by Chi-square and Kruskal-wallis ($P < 0.05$).

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Identification of three spermatozoa subpopulations in crossbred bulls (*Bos taurus taurus x Bos taurus indicus*)

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Introduction

Sperm subpopulations in the bull can be determined by different motility and morphometric characteristics of the cells present in an ejaculate and were previously described by Quintero- Moreno et al. (1). Computer assisted semen analysis (CASA) is a technique used to identify traits such as linearity index and amplitude of lateral head displacement of sperm cells and also to quantify spermatozoa motility patterns, like percentage of motile cells and average of curvilinear velocity. Morphological aspects of the cells can be assessed by computerized methods like automated sperm morphology analysis (ASMA) and possibly by other software. The aim of this work was to assess the morphological characteristics of the bull sperm cells using the free software IMAGE J® identifying different cell clusters, whether they are present.

Material and Methods

Semen samples of six crossbred donor bulls (*Bos taurus taurus x Bos taurus indicus*) were collected by eletroejaculation and fixed in buffered-phormol-saline solution before further preparation of smears. For cell image acquisition it was used a microscope (*Olympus BX41*) with a green filter (*Olympus IF 550*) connected to a charge coupled detector (CCD) camera (*Samsung SPC-415*) and a computer for image storage in a ".bmp" format. The acquired images (n = 40 for each bull) were converted to 8 bits files and were treated by the IMAGE J® tools "*Threshold*" and "*Wand (tracing) tool",* in order to enhance quality and to eliminate background unspecific signals. For each head of the cell the parameters evaluated were the area, perimeter, major and minor axis of the ellipsis and the center of mass (XM and YM). Only cells considered to be normal in shape were evaluated. Data were analyzed among the different semen donors and among cells of the same ejaculate of each bull. For statistical analysis, with a nominal significance level of 5%, the SISVAR software was used.

Results and Discussion

Preliminary results showed that there were three distinct morphological subpopulations (clusters) of cells according to their size (great, intermediate and small cells) present in the semen samples of each bull. Among bulls, differences were observed in area, perimeter and major and minor axis of the ellipses ($P < 0.05$), indicating subtle variations in the cell morphology of different semen samples identified by the IMAGE J® tools. The mass center (XM and YM) is an estimate of the IMAGE J® that shows the degree of shape disruption in the head of the sperm cell. Among bulls, no differences were found ($P > 0.05$) in this parameter, indicating that the cells exhibited uniformity and no pathological conditions were present in order to alter it. In conclusion, in crossbred bulls the semen showed cells with distinct morphometric properties and the spermatozoa could be separated in at least three different clusters. The free software IMAGE J® allows sperm cell morphological assessment and should be considered as an improving toll in semen evaluation.

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Evaluation of MPF and MAPK activities in recipient bovine oocytes produced by chemical enucleation techniques in nuclear transfer

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Introduction

Although there are reports of advances in nuclear transfer (NT) in recent years, this procedure still presents low efficiency. Several studies employing different mammalian species have demonstrated a key effect of the cell-cycle stage of both the donor nucleus and the recipient cytoplasm on the developmental potential of reconstructed embryos. Within this context, maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) are very important for mammalian preimplantation development. The aim of the present study was to compare the MPF and MAPK activities between oocytes subjected to the chemically assisted or induced chemical enucleation techniques, using histone H1 and myelin basic protein (MBP) for a double assay.

Materials and Methods

Oocytes obtained from cow ovaries collected at a slaughterhouse were IVM in TCM 199 medium supplemented with 10% FCS, FSH, hCG, estradiol, pyruvate and amikacin for up to 26 h. In technique 1 (chemically assisted enucleation), oocytes *in vitro* matured for 19 h were denuded with hyaluronidase and exposed to 0.05 mg/ml demecolcine for 2 h to form a membrane protrusion containing a condensed chromosome mass. In technique 2 (induced chemical enucleation), oocytes were denuded and artificially activated after 26 h of IVM with 5μM ionomycin for 5 min and 10 μg/ml cycloheximide for 4 h. After 2 h of activation, the oocytes were exposed to demecolcine to complete 4 h of activation, aiming to extrude all nuclear material with the 2nd PB. Groups of four oocytes were collected in three replicates at different times: technique 1 – MII oocytes (19 h IVM), and 0.5 h, 1 h, 2 h, 4 h, and 8 h after demecolcine exposure; technique 2 – MII oocytes (26 h IVM), and 0.5 h, 1 h, 2 h, 3 h, 4 h, and 8 h after the beginning of activation. The samples were analyzed for histone H1and MBP activities as previously described by Christmann et al. (1) and Kubelka et al. (2). We arbitrarily set the activity in MII oocytes as 100% and the other values were expressed relatively to this activity. The results obtained were submitted to ANOVA and the means were compared by Tukey test using the SAS system at the 5% significance level.

Results and Discussion

Regarding the histone H1 kinase activity in technique 1 it was observed that the means of the group exposed to demecolcine were similar to the control group (without demecolcine treatment) during evaluation, ranging from 96.3% to 165.4% during the first 4 h. We observed superior values only at the final assessment (~ 181%), but without differences between groups. The MBP kinase had a similar behavior of its activity, with means ranging from 81.8 to 103.5%. In the second technique, histone H1 kinase declined after 0.5 h of activation (70.3% of activity), and further decrease was observed after 2 h (39.1%). Nonetheless, treated and control groups showed similar means at all times during the investigation. On the other hand, the MBP kinase activity declined only after 8 h (means from 40.2 to 58%), despite no differences between groups. The destruction of spindles by demecolcine inhibits degradation of cyclin B (3), which leads to an increase in MPF activity. Nonetheless, the present study concludes that this effect is presumably dependent on demecolcine concentration, since MPF and MAPK were not affected by chemical processes when demecolcine was used in low concentration (0.05 μg/ml).

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α-6 integrin expression in bovine spermatogonial cells purified by discontinuous Percoll gradient

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Introduction

The study of spermatogonial stem cells (SSCs) provides a model to better understand adult stem cell biology. Besides the biomedical potential to perform studies of infertility in many species, SSC hold a promising application at transgenesis. Because stem cells are thought to be associated with basement membranes, expression of α-6 integrin has been investigated as a marker of type A spermatogonial cells, which are considered SSCs due to their undifferentiated status. In this manner, the aim of this study was to isolate type A SSCs from adult Nelore bulls by a two-step enzymatic procedure (1) followed by a discontinuous Percoll density gradient purification (2) and verify the expression of α-6 integrin by flow cytometry before and after Percoll purification.

Materials and Methods

Spermatogonial cells were isolated from abattoir testes of 6 adult Nelore bulls by a two-step enzymatic procedure. Briefly, fragments from each testis were finely chopped in DMEM with $NaHCO_3$, HEPES, L-glutamine, non-essential aminoacids and antibiotics and ground in a tea strainer with a syringe plunger. The remaining tubule sections were digested twice in DMEM with collagenase, hyaluronidase and trypsin at 32°C for 45 min. Cells were cultured in DMEM with 10% (v/v) FCS overnight at 37°C in a high humidity 5% (v/v) CO_2 in air atmosphere. Cell viability was assessed by trypan-blue exclusion. Non-adherent cells (approximately 5 x 10^6 cells) were poured over a Percoll density gradient (1.0611, 1.0542, 1.0513 and 1.0413 g/mL) and centrifuged at 800 x g for 30 min. Samples were collected before (Fraction 0) and after Percoll purification (Fractions 1-4) and fixed in cold 70% (v/v) ethanol. Fixed cells were incubated with PBS with 10% (v/v) FCS, 1% (w/v) sodium azide, PI and FITC-conjugated anti-α-6 integrin antibody (1:10) at 4°C for 30 min. Negative control was prepared with cells incubated without antibody. Cells were analyzed using the Guava Easy Cyte Mini Base System (Guava Technologies, Hayward, CA, USA). The percentage of α-6 integrin-positive cells was analyzed by parametric ANOVA (LSD test) using *Statistica for Windows* software at a 5% level.

Results and Discussion

Spermatogonial cells were successfully obtained using the two-step enzymatic digestion. An average of 8x105 viable cells per g of testes was isolated. However, the discontinuous Percoll did not purify isolated cells regarding α-6 integrin expression. Flow cytometry analysis demonstrated no differences in the α-6 integrin expression between cell samples before and after Percoll purification (p = 0.5636; Fig. 1). Considering that Percoll can reduce cell viability, it is possible to conclude that Percoll density gradient is not suitable to purify bovine SSC, according to α-6 integrin expression. In addition, this study is the first report to verify the α-6 integrin expression in bovine spermatogonial cells.

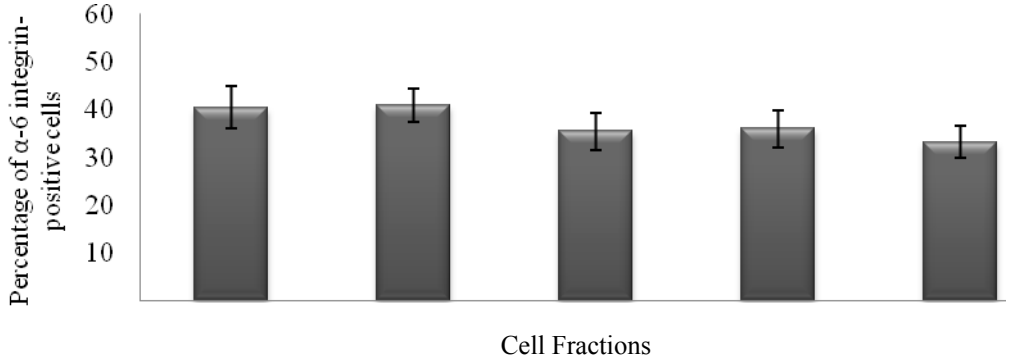

Figure 1. Percentage of spermatogonial cells expressing α-6 integrin assessed by flow cytometry, before and after Percoll gradient purification (LSmeans ± SEM).

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In vitro viability of ram sperm frozen with quercetin

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Introduction

Although sperm cryopreservation generate large benefits to animal production, this biotechnology induces extensive cryoinjuries responsible by sperm fertility reduction, with emphasis on the generation of reactive oxygen species (ROS), that result in oxidative damage and decreased sperm function (1). Thus, because quercetin is a flavonoid polyphenol with powerful antioxidant activity (2), the aim of this study was to evaluate the effect of different concentrations of quercetin on *in vitro* viability of ram sperm.

Material and methods

Four Santa Inês crossbred males were subjected to semen collected (n = 6) with artificial vagina. After collection, semen samples of each ram formed a *pool* of semen, which was evaluated, diluted in Tris yolk-egg containing 5% glycerol with or without quercetin (0, 5, 10, 15 and 20 µg/ml, respectively, Q0, Q5, Q10, Q15 and Q20), packaged in straws (0.25 ml; 100 x 10^6 spermatozoa) and frozen. The thawed semen samples (37°C/30 seconds) were evaluated for progressive motility (PM), vigor, plasma membrane integrity (PMi) with CFDA and PI, acrosome integrity (ACi) with FITC-PNA and mitochondrial membrane potential (MMP) with JC-1. Data was analyzed by analysis of variance (ANOVA) and Tukey test at 5%.

Results and Discussion

No significant difference was observed ($P > 0.05$) between groups in PM, vigor, PMi and ACi, although these parameters have been normal for all groups (Tab. 1). On other hand, the semen sample of Q0 group showed higher ($P < 0.05$) percentage of MMP than Q10, Q15 and Q20, as well as Q5 was higher ($P < 0.05$) than Q15 and Q20, where the MMP reduction was considered proportional to the increase of quercetin concentration. Despite the fact that quercetin does not preserve the viability of semen samples in the treated groups, with the exception of MMP, almost all group had numerically higher value of spermatozoa with PM (Q5, Q10 and Q20), vigor (Q5, Q10, Q15 and Q20), PMi (Q5, Q10 and Q15) and ACi (Q5, Q10, Q15 and Q20) than control group (Q0). Although these results did not show significant differences between groups, they characterized the ROS production during freezing process and the beneficial effect of quercetin addition on these parameters of ram sperm. Therefore, it is possible that the MMP reduction has been determined by the antioxidant quercetin, which acts as a ROS scavenger and metal chelator, while the mitochondria are the principal source of intracellular ROS (1) and the respiratory chain may act in the oxidation process (3). So, it can be concluded that other studies should be conducted to evaluate other concentrations of this antioxidant and its effect on fertility rate of ewes artificially inseminated.

Table 1. Spermatic parameters of ram semen frozen with different concentrations of quercetin

Spermatic parameters	Experimental groups				
	Q0	Q5	Q10	Q15	Q20
PM (%)	50.83 ± 2.04	53.33 ± 10.80	53.33 ± 8.76	45.00 ± 4.47	55.00 ± 8.37
Vigor (0-5)	3.08 ± 0.20	3.50 ± 0.55	3.50 ± 0.55	3.33 ± 0.52	3.83 ± 0,41
PMi (%)	36.25 ± 3.28	39.75 ± 10.24	39.17 ± 9.65	37.33 ± 5,72	35.42 ± 4.44
ACi (%)	62.25 ± 7.04	66.33 ± 3.72	64.50 ± 5.41	67.75 ± 9.45	64.33 ± 6.22
MMP (%)	36.25 ± 8.12^{a}	20.58 ± 12.05^{ab}	9.33 ± 6.54^{bc}	6.25 ± 5.98^{c}	5.17 ± 5.08^{c}

Different letters in the same line $P < 0.05$.

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Effect of Megalac-E® supplementation on pregnancy rate of embryo recipients submitted to FTET or natural heat

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Introduction

Lipids are an important source of energy to animals in addition to being a structural component of the cell membrane and acting in physiological activities indispensable to the cells. Some studies have shown positive effects of polyunsaturated fatty acids (PUFA) in reproduction of bovine, however, the mechanism related to the effect of fatty acids on oocyte quality and embryo are not well understood (1,2). Staples et al. (3) reported that the fat supplementation improved the conception rate of dairy cows and the development of follicles during the estrous cycle.

Material and Methods

Two experiments were conducted to evaluate the effect of Megalac-E supplementation on pregnancy rate of crossbred heifers submitted to natural heat embryo transfer or fixed-time embryo transfer (FTET). In both experiments, cows were allocated in one of two treatments: Control and Megalac-E groups. Coes in the Control Group received mineral, protein and energetic supplementation. In Megalac-E Group, cows received the same treatment plus 100g of Megalac-E (Arm & Hammer, a Church & Dwight Company, Brazil). On Experiment 1 (FTET), 191 embryo receptors were synchronized with the following protocol: D0-EB+P4; D8-eCG+PGF+EC+P4 withdrawal; D15-FTET. On Experiment 2 (natural heat), 291 embryo recipients were used. Treatments were given every other day from the beginning of protocol (Experiment 1) or estrus observation (Experiment 2) for a 60 days period. Cows were allocated to a rotational grazing system and the cows of Control and Megalac-E groups rotated on the same pastures during the experiment. Pregnancy diagnosis was performed by ultrasonography 30 and 60 days after embryo transfer. The statistical analysis was performed using GLIMMIX procedure of SAS.

Results and Discussion

There was no difference on conception rates between treatment 30 (P = 0.19) or 60 days (P = 0.11) after embryo transfer, both on FTET and in natural heat. There was, however, a numerical reduction on pregnancy loss on cows treated with Megalac-E.

Table 1. Effect of Megalac-E® supplementation on pregnancy rate of embryo recipients, 2008.

Rate (%)	Control	Megalac-E	P
Pregnancy 30 days	27.5 (66/240)	33.1 (80/242)	0.19
Pregnancy 60 days	22.5 (54/240)	28.9 (70/242)	0.11
Pregnancy loss	18.2 (12/66)	12.5 (10/80)	0.46

Table 2. Effect of Megalac-E® supplementation on pregnancy rate at 30 days of embryo recipients submitted to FTET or natural heat, 2008.

	Control	Megalac-E	P
Natural heat	25.0 (36/144)	31.3 (46/147)	0.23
FTET	31.3 (30/96)	35.8 (34/95)	0.54

We conclude that Megalac-E does not increase the conception rate of embryo recipients. However, it was observed an increase of 6% on conception rate in cows treated with Megalac-E.

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Use of medium supplemented with FCS and knockout serum replacement® for derivation of mouse embryonic stem cell from C57BL/6/EGFP

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Introduction

Embryonic stem cells (ESC) have been used in attempts to obtain specific tissues or even individuals. Those cells are pluripotent, allowing the differentiation of cell types from three germ layers. The establishment of a stable lineage of ESC is a valluable tool; however, there are strains of mice less permissive to ESC derivation and/or generation of chimeric animals (*e.g.*, C57BL/6). To ESC derivation the supplementation of culture medium with FCS may influence the effectiveness to derivation and/or the use of those strains in tetraploid complementation assays (1). Thus, serum replacement was carried out using knockout serum replacement (KSR®) to minimize the potential deleterious effect of the serum (2). The establishment and characterization of pluripotency of those cells could be an important model since C57BL/6 is the most used genetic background for phenogenomic studies.

Materials and Methods

Eight embryos were obtained from five superstimulated females (3) of the lineage C57BL6/EGFP, aged between 21 and 30 d and weighting approximately 35 g. The animals were placed to mating – with fertile males of the same strain – in a proportion of 1:1 (male: female). The copulation was confirmed by detection of the vaginal plug. Embryo recovery was performed on days 3.5 to 4.0 dpc to obtain expanded (BX) and/or hatched blastocysts (BE). Zona pellucida was removed from BX embryos with the aid of pronase solution and the embryos were deposited on 4 wells dishes pretreated with 0.1% pig skin gelatin, under MFP (fibroblast primary murine) in DMEM medium supplemented with 7.5% FCS and 7.5% KSR®, 10mM βmercaptoetanol, 1mM sodium pyruvate, 2mM L-glutamine and 83.4 mg/mL amikacin for 24 h. After this period, the medium was replaced by DMEM supplemented with 15% KSR®.

Results and Discussion

Only one colony grew between 3 and 6 days after culture of embryos. Once established, the colony was picked and placed into new plates containing MFP every 48 to 72 h. After 14 d, the presence of pluripotency markers was confirmed in the cells by immunofluorescence for Oct3/4, Sox2 and Nanog, besides karyotyping for ploidy detection. The reaction was positive for all tested markers, in addition to the detection of the endogenous fluorescence of EGFP protein itself (strain origin). Following the rules to name new ESC lineage that one was called BCM04. In less permissive strain (as C57BL/6) ESC derivation with serum replacement was feasible, although with a reduced derivation rate (12.5%, *i.e.*, 1 lineage from 8 attempts).

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Effects of estradiol benzoate or GnRH at the beginning of a FTAI protocol on ovarian follicular diameter and pregnancy rate in postpartum beef cows

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Introduction

Anestrus and weak estrus signs in cows that are cycling result in low reproductive efficiency in postpartum beef cows (1). Hormonal treatments that allow fixed-time artificial insemination (FTAI) can be used to alleviate these problems and increase efficiency. Estradiol benzoate (BE) in combination with progesterone suppresses the development of the dominant ovarian follicle and results in the synchronized emergence of a new follicular wave 4.3 days after treatment (2). GnRH causes ovulation or atresia of the dominant follicle depending on the stage of follicular growth resulting in the emergence of a new follicular wave two days after treatment (3). The objective of the present study was to determine the effect of BE or GnRH administered concomitantly with a progesterone implant at the beginning of a FTAI protocol on ovarian follicular diameter and pregnancy rate in postpartum beef cows.

Materials and Methods

Nelore cows 36 to 60 months old, 34 to 90 days postpartum, and with a mean body condition score of 3 (range 1-5) were used in this study. The cows were kept in *Brachiaria brizantha* pasture and received mineral supplementation *ad libitum*. Cows were divided randomly in two groups: G-BE (n = 32) and G-GnRH (n = 29). At the beginning of the FTAI protocol (D0) cows received a progesterone implant (CIDR®) and 2 mg BE, IM (Estrogin®; 0.005g/5 ml; G-BE) or 2.5 ml GnRH, IM (Conceptal®; G-GnRH). On D9 the progesterone implant was removed, and all cows received 2.5 ml PGF2α, IM (Lutalyse®, 5 mg/ml) and 0.25 ml estradiol cypionate (E.C.P.®, 2 mg/ml), IM followed by removal of the calves. On Day 11 (48 hours after implant removal) all cows were inseminated and the calves returned. On D9 the diameter of the dominant follicle was measured by ultrasonography. Pregnancy rates were determined by ultrasonography 30 days after insemination.

Results and Discussion

Although there was no significant difference in pregnancy rates, G-EB showed a pregnancy rate 17% greater than G-GnRH (57.5 and 40.5%, respectively). The lower pregnancy rate in GnRH group might have been the result of a longer period of follicular growth resulting in a greater proportion of LH-unresponsive follicles (i.e. late static or regressing) when ovulation was induced with estradiol. According Barros et al. (4), a new follicular wave emerges two days after GnRH treatment and approximately five days after BE treatment (2.) Therefore, dominant follicles in the G-GnRH likely emerged three days before dominant follicles in the G-BE group and were "older" at the time of implant removal and estradiol treatment. Follicular diameter on D9 was greater ($p < 0.05$) in pregnant (10.7 mm) than in non-pregnant (8.5 mm) cows in the G-EB. However, there was no difference on follicular diameter between pregnant (11.6 mm) and non-pregnant (10.2 mm) cows in the G-GnRH. In conclusion, the use of GnRH on D0 did not improve pregnancy rates in postpartum beef cows even though it resulted in larger follicles at the time of induction of ovulation. Therefore, it is necessary to take into account the cost of the drugs used in FTAI protocols.

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Timed-AI efficiency of a progesterone-based synchronization protocol using GnRH (Co-synch) or estradiol in *Bos indicus* beef heifers

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Introduction

It was previously shown that the 5-day Co-Synch+CIDR (controlled internal drug-release) protocol would be an acceptable reproductive management program for timed artificial insemination (TAI) of beef and dairy *Bos taurus* cattle (1). However, it is no clear if this strategy works well in *Bos indicus* cattle, which is known to show some physiological differences compared to *Bos taurus* cattle. This study aimed to evaluate conception rates of Nelore (*Bos indicus)* beef heifers using a progesterone-based synchronization protocol associated to the 5-day Co-Synch or estradiol conventional treatment.

Material and Methods

At Day 0, post-pubertal Nelore heifers (n = 90) were treated with a progesterone intravaginal device (Primer ®, Tecnopec, São Paulo, Brazil) containing 1 g of progesterone and assigned randomly in two groups. Animals on Group 1 (n = 45) were injected with GnRH (100µg i.m. Fertagyl®, Intervet) and on Group 2 (n = 45) with estradiol benzoate (2mg EB, Estrogin, AUSA, Brazil). Primer was removed on Day 5 (Group 1) and Day 8 (Group 2) and animals received an injection of cloprostenol (125 mcg, Prolise®, Tecnopec, São Paulo, Brazil). Twenty-four hours later, Group 1 heifers received a second injection of cloprostenol while Group 2 received 2 mg EB. Heifers were TAI (semen from one sire) 72 (Group 1) or 54 (Group 2) hours after Primer removal and GnRH was administered concurrently with TAI in Group 1. Estrus was monitored after cloprostenol injection and pregnancy status was evaluated via transrectal ultrasonography on day 40 after TAI. Data were analyzed by Chi-square test.

Results and Discussion

Timed AI pregnancy rates for the two treatment groups are summarized in Table 1.

Table 1. Reproductive performance of Nelore beef heifers receiving either 5-day Co-Synch (Group 1) or EB (Group 2) treatments in a progesterone-based synchronization protocol.

Variable	Co-Synch			EB		
	Estrus	No estrus	Total	Estrus	No estrus	Total
n	5	40	45	20	25	45
Pregnancy	4	12	16	8	8	16

Estrous response was lower ($P < 0.05$) in Group 1 (55%) than in Group 2 (83%). However, no difference in conception rate was detected between Group 1 (35.6%) and Group 2 (35.6%). In a previous study with lactating Holstein cows, Kim et al. (2) reported a pregnancy rate significantly higher in the Co-Synch group (65.0%) than in the EB group (35.0%) and this difference was related to ovulation of significantly smaller dominant follicles in animals receiving the EB treatment. Therefore, more research is needed to elucidate the factors (breed, parity, etc.) responsible for these differences.

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Influence of refrigeration of bone marrow on the viability of mesenchymal stem cells

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Introduction

The interest in the application of cell therapy with stem cells has become an important tool in the treatment of several pathologies (3). Bone marrow has a type of stem cells called mesenchymal stem cell that has a high ability to renew and differentiate into several lineages of connective tissue (1). Cell therapy aims the recovery of damaged tissues and organs through the introduction of new cells, which are able to colonize affected areas and restore the function previously lost (3). This therapy allows the treatment of degenerative and congenital diseases, trauma and injuries of different etiologies (2, 4). The current study has the objective to evaluate the influence of refrigeration on the viability of mesenchymal stem cells from bone marrow.

Materials and Methods

For this experiment, the culture of stem cells was evaluated in six different times of refrigeration before and after culture: without refrigeration; 3 hours of refrigeration; 6 hours of refrigeration; 9 hours of refrigeration; 12 hours of refrigeration; 24 hours of refrigeration. Bone marrow aspiration was performed in eight undefined breed mares with age between 4 and 23 years old with good body condition. The mares were previously sedated with Detomidine and had the region of the fifth sternebrae tricotomized and then a local anesthetic infiltration was performed with lidocaine chloridrate. Bone marrow aspiration was made with an 8 gauge x 12cm length *Jamishidi* 6 needle and a 20 ml syringe containing 5ml of DPBS and 1ml of heparin. Subsequently, the samples were filtered with a catheter for blood transfusion and centrifuged. The supernatant was removed and the pellets were ressuspended 1:1 in DMEN High Glucose. Then, the bone marrow cells were separated through centrifugation with the Ficoll gradient, and the halo was washed with DMEN High Glucose. The supernatant was removed and the pellets were ressuspended in DMEN High Glucose with 20% Fetal Bovine Serum; 3µg/ml amphotericin and penicillin 100 UI/ml. The samples were packaged in 15ml tubes, labeled with the name of the animal and then stored in a container at 14^{o}C. The culture was performed according with the times of refrigeration and then after this procedure the mesenchymal stem cells were refrigerated again in different times. The viability of mesenchymal stem cell was evaluated by morphological characteristics using an inverted microscopy.

Results and Discussion

As seen in Table 1, stem cells from bone marrow demonstrated a great development of mesenchymal stem cells without refrigeration, however there was a significant decrease in the viability of refrigerated cells after the culture. Based on the results of the present experiment we can conclude that the refrigeration of bone marrow to 14^{o}C is viable prior to culture, and can be used to transport samples from the field to laboratory for the culture of mesenchymal stem cells. However, there was a gradual decrease in the viability of mesenchymal stem cells after the culture along the 24 hours of refrigeration ($P < 0.05$).

Table 1. Viability of mesenchymal stem cells before and after culture in different times of refrigeration.

Refrigeration	PRE-CULTURE		AFTER-CULTURE	
(hours)	Living cells (%)	Dead cells (%)	Living cells (%)	Dead cells (%)
0	97^{A}	03	97^{A}	03
3	95^{A}	05	80^{AB}	20
6	97^{A}	02	76^{BC}	24
9	94^{A}	06	74^{C}	26
12	92^{A}	08	68^{C}	32
24	97^{A}	03	54^{D}	46

Within the same column, values without common superscripts letters (A-D) differ significally ($P < 0.05$).

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Effect of addition of the antioxidants superoxide dismutase (SOD) and reduced glutathione (GSH) to the extender for cryopreservation of ram semen

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Introduction

Small amounts of reactive oxygen species (ROS) are required to initiate sperm functions, such as capacitation and induction of acrosome reaction (1). However, it is necessary to maintain a balance between ROS production and antioxidant capacity on the environment to ensure viability of gametes (2). It is known that processes of cryopreservation are associated with reduced levels of glutathione (3) and superoxide dismutase (4), probably caused by semen dilution, which determine increase of ROS production and consequently injuries to sperm cells (5). The objective of this study was to evaluate the effect of addition of the antioxidants SOD and GSH to the extender for cryopreservation of ram semen.

Material and Methods

Five Santa Inês breed rams with history of fertility were used and ejaculates was obtained by artificial vagina. After collection, a pool of semen samples of each ram was diluted to a final concentration of 240 x 10^6 spermatozoa/ml in Tris egg-yolk extender containing 5% glycerol supplemented with antioxidants (G1= Control; G2= 25 U/ml SOD; G3= 50 U/ml SOD; G4= 100 U/ml SOD; G5= 2 mM GSH; G6= 5 mM GSH; G7= 7 mM GSH). Semen samples were packed in straws (0.25ml), frozen using an automated system and stored in liquid nitrogen (-196ºC). After thawing (37ºC/30 second), semen samples were subjected to analysis of plasma membrane (iMP) and acrosomal integrity (iAc), mitochondrial membrane potential (MMP), kinematics and ultrastructure of spermatozoa. *In vivo*, the semen was used in embryo transfer program. Data were analyzed by ANOVA and group means were compared using the Tukey-Kramer ($P < 0.05$).

Results and Discussion

In the evaluation of post-thawing semen, there were no significant differences between groups on the iMP, iAC and PMM results. Significant differences ($P < 0.05$) were observed among groups for the total motility (MT), straightness (STR) and wobble (WOB), where GSH 7 mM group had lower MT and higher STR than the control group. GSH 5 and 7 mM groups had higher wobble than control, SOD 25 and 100 U/ml groups. The ultrastructural analyses showed that acrosome was better preserved ($P < 0.05$) after freezing in SOD (50 and 100 U/ml) and GSH (5 and 7 mM groups) than control the group, that showed disruption and loss of acrosome. Mitochondria from the control group and GSH 7 mM suffered more damages, as mitochondrial vacuolation, than other groups. On the *in vivo* fertilization, the SOD (100 U/ml; 87.5%) group had higher fertilization rate than control (66.67%) and GSH (2 mM; 58.34%) groups. According to the results, it can be concluded that addition of GSH 7 mM does not preserve the integrity of ram spermatozoa post-freezing, while the addition of SOD 100 U/ml in Tris egg-yolk extender protects sperm cell membrane during the freezing process.

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Cryopreservation of dog semen in 0.5ml straw and Flat-bag

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Introduction

Cryopreservation is a process that could impair at least 50% of cells (1). Focusing on the reduced damage on frozen/thawed spermatozoa, some authors used different packaging, for example: pellet (2, 3), 0.25 and 0.50 ml straws (2, 3), 5.0 ml Maxi-straws or 5.0 ml aluminum tube (4), and plastic flat-pack (5,6). Recently a new Mini-flatpack presented better performance than 0.5 ml straws, in post-thawing (5, 7, 8). It occurs due to fast heat exchange and uniform ice formation in semen samples, frozen in flat-bags (9), or to high heat dissipation during free water phase changes compared with straws (10). The aim of this study was to evaluate the performance of flat-bags compared to straws for canine semen freezing.

Material and Methods

Ten ejaculates from different dogs were collected. Aliquots of 0.5 ml of semen diluted in Tris-yolk-glucose were transferred to 0.5 ml straws or 4.0 x 3.0 cm flat-bags made with polypropylene of low density (Ziploc®). Both packages were frozen and later thawed. The motility evaluation was made by Hamilton Throne Research 0, 5, and 20 minutes after thawing. The integrity of plasma membrane was evaluated by HOST, immediately after thawing the semen. Statistical analysis was performed by Sisvar® software.

Results and Discussion

Total and progressive motility were unaltered between straw 0.5 ml and flat-bag (Table 1; $P > 0.05$), despite a numerical increase of the mean value of these variables in semen frozen in flat-bags.

Table 1. Total motility and progressive motility of dog semen after thawing (mean ± SEM)

Variables	Time(min.)	Straw	Flat-bag
Total motility	0	43.3 ± 4.2	50.4 ± 5.3
	5	45.5 ± 4.9	51.9 ± 4.8
	20	37.7 ± 4.7	49.4 ± 6.9
Progressive Motility	0	27.5 ± 2.8	32.4 ± 3.8
	5	31.4 ± 4.0	33.4 ± 3.1
	20	23.3 ± 3.7	30.9 ± 4.8

Similar results were found for Ekwall et al. (6), which compared the cryopreservation of boar semen in straw or FlatPacks. No significant differences were found comparing FlatPack with Max-straw (5), for boar semen thawed at 35, 50 or 70°C. The authors also stated that the high thawing temperature was related to high motility. Plasma membrane integrity, using the HOST, was not different between the packaging systems. In conclusion, Flat-bag seems to be better a package than straws, but fertilization tests should be performed.

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Parameters for eletroporation of bovine oocytes to facilitate the introdution of trehalose in the cytoplasm

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Introdution

The results of cryopreservation of bovine oocytes are still very low (1). An alternative to improve these results is the use of less toxic cryoprotectants, including trehalose. However, the fact that sugars are impermeable to the plasma membrane, it is necessary to develop techniques that allow its introduction into the ooplasm (2). Electroporation can be a practical alternative, because it promotes the opening of transient pores in the plasma membrane of this gamete. In this sense, the study aimed to evaluate different parameters for electroporation of bovine oocytes and the effects of these treatments on oocyte viability.

Material and Methods

One hundred *cumulus oophorus* complexes obtained from slaughterhouses were *in vitro* matured and denuded and electroporated in plate (450, BTX), in 20 µl of electroporation solution (0.1 mM magnesium sulfate, 150 mM trehalose and 140 mM mannitol). Different parameters were compared: T1 (4 pulses of 2 µs each, 40 V) and T2 (130 V and 5 pulses of 60 µs each). After electroporation the oocytes were labeled with propidium iodide (PI) (10 µg/ml in PBS) and observed at to 5 and 30 minutes. It was expected that at five minutes the cells should present opened pores in the membrane and at 30 minutes pores should be closed.To access cell viability, oocytes were labeled with calcein AM (1 mg/ml), 30 minutes after the electroporation.

Results and Discussion

Five minutes after the electroporation, the oocytes of T1 and T2 showed a significant difference in the percentage of PI (75 ± 1.5 and 100 ± 3.1, respectively). However after 30 minutes the oocytes from both treatments showed no significantly different results (15 ± 3.1 and 35 ± 3.1 respectively).The assessment of cell viability by marking with calcein AM showed that oocytes from T1(84% ± 0.3) showed greater viability than those of T2(43.1 ± 0.5) according to confidence interval proportion test ($P < 0.05$). The results presented indicate a higher rate of plasma membrane permeabilization promoted by treatment 2. But the reduction in voltage, as used in T1, allowed an increase on the perrmeabilization of the membranes of oocytes because of the lower impact on its viability.

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Effects of embryonic fluid and serum replacer as protein sources for *in vitro* maturation of bovine oocytes

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Introduction

In vitro embryo production (IVEP) is influenced by the culture conditions during *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC). In the present study, we evaluated for the first time two protein sources in IVM: embryonic fluid (EF) and serum replacer (SR). The objective was to evaluate the effects of replacement of bovine-derived blood proteins (FCS and BSA) with EF and SR during IVM on oocyte maturation and embryo development. To address this question, nuclear and cytoplasmic maturation, embryo cleavage rates, blastocyst development and hatching rates were carefully assessed.

Material and Methods

Oocytes obtained from cow ovaries collected at an abattoir were IVM in TCM 199 medium supplemented with FSH, hCG, estradiol, pyruvate, amikacin and 10% FCS (group control), or 10% EF (E1761 Sigma - EF group) or 20 µL/ml SR (S0638 Sigma - SR group) for up to 24 h. IVF was performed in mineral oil-covered droplets of 100-µl TALP-IVF medium (TALP medium supplemented with 30 µg/ml heparin, 18 µM penicillamine, 10 µM hypotaurine and 1.8 µM epinephrine) containing 0.2 mM pyruvate and 83.4 µg/ml amikacin, and supplemented with 6 mg/ml BSA. After IVF, presumptive zygotes were cultured in 4-well dishes containing 500 µl synthetic oviductal fluid (SOF) supplemented with 10% FCS and 4 mg/ml BSA under mineral oil. Oocyte maturation was evaluated based on the stage of nuclear progression (MII stage) and cortical granule migration to the periphery of the cytoplasmic membrane at the end of IVM. Briefly, cumulus cells were removed with 0.2% hyaluronidase and the zona pellucida was removed with acid solution, pH 2.5. The oocytes were then fixed in 3% formaldehyde and incubated in blocking solution overnight at 4ºC. For permeabilization, the oocytes were treated with 0.1% Triton X-100 and incubated with 10 µg/ml fluorescein isothiocyanate-conjugated *Lens culinaris* agglutinin and 10 µg/ml Hoechst 33342. The oocytes were then mounted on slides and examined under an Olympus IX-FLA-70 epifluorescence microscope (Tokyo, Japan) for the evaluation of meiotic progression and the distribution of cortical granules. Images of each oocyte were captured with an AxioCam camera and stored using the AxioVision 4.7.1 software (Carl Zeiss, Jena, Germany). The cleavage rate was determined approximately 32 to 36 h post-insemination under a stereomicroscope (magnification of 50X). Cleavage was defined as the presence of two- to four-cell embryos. Blastocyst development and hatching rates were evaluated on days 7 and 8 after IVF, respectively. The results obtained were submitted to ANOVA and the means were compared by Tukey test using the SAS at 5% significance level.

Results and Discussion

There was no difference ($P > 0.05$) between EF group (81.98%) and control group (85.74%) on nuclear progression to MII stage. However, SR group (77.54%) was inferior ($P < 0.05$) to the control group. For IVM, EF (70.95%) and SR (70.03%) groups resulted in similar ($P > 0.05$) cortical granule migration to the periphery of the cytoplasmic membrane when compared to the control group (73.35%). Cleavage rates were similar ($P > 0.05$) among EF (83.85%), SR (79.44%), and control (85.88%) groups. Blastocyst development was inferior ($P < 0.05$) in SR group (38.05%) when compared to control and EF groups (55.41 and 50.98%, respectively). For hatching rates, treatments using EF (41.86%) and SR (40.02%) were similar ($P < 0.05$) to control group (41.45%). In this preliminary study it is possible to conclude that EF and SR are suitable proteins sources to replace FCS and BSA in IVM for bovine *in vitro* embryo production.

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The role of insulin-like growth factor-I on inhibition of heat-induced apoptosis in bovine oocytes

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Introduction

Infertility caused by environmental heat stress is one of the most important economic problems facing the dairy industry. Rectal temperatures in heat-stressed dairy cows may exceed 41°C (1). It has been demonstrated that oocytes and early preimplantation bovine embryos are affected by the deleterious effects of heat stress. Indeed, culture of oocytes during maturation at elevated temperatures can induce oocyte apoptosis and reduce subsequent cleavage rate and blastocyst development following in vitro fertilization (2, 3, 4, 5). Therefore, the objective of the current study was 1) to determine the effect of elevated temperature on heat-induced apoptosis in bovine oocytes and 2) to evaluate whether insulin-like growth factor-I (IGF-I) exerts a thermoprotective effect on bovine oocytes subjected to heat shock.

Materials and Methods

Slaughterhouse derived cumulus-oocyte complexes (COCs) were subjected to Control (38.5°C for 22 h) and Heat Shock (41°C for 14 h followed by 38.5°C for 8 h) treatments in the presence of 0 or 100 ng/ml IGF-I during in vitro maturation (IVM). Approximately 22 h following IVM oocytes were mechanically denuded by repeated pipetting and fixed in 4% paraformaldehyde until TUNEL (Terminal deoxynucleotidyl transferase nick-end labeling) analysis. The TUNEL procedure was used to detect DNA fragmentation observed in late stages of apoptosis. The enzyme terminal deoxynucleotidyl transferase is a DNA polymerase that catalyzes the transfer of a fluorescein isothiocyanate-conjugated dUTP nucleotide to a free 3' hydroxyl group present in DNA strand breaks. Data were analyzed by least-squares analysis of variance using the General Linear Models procedure of SAS.

Results and Discussion

The proportion of TUNEL-positive oocytes was affected by Temperature ($P < 0.01$) and IGF-I ($P < 0.05$). There was a Temperature x IGF-I interaction ($P = 0.06$). In the absence of IGF-I the proportion of TUNEL-positive oocytes increased from 34.05 ± 6.3% to 75.04 ± 6.3% in the Control versus Heat Shock group, respectively. However, the magnitude of such deleterious effect of heat shocked was reduced by IGF-I. In the presence of IGF-I the proportion of TUNEL-positive oocytes increased from 28.63 + 6.3% to 45.25 + 6.3% in the Control-IGF versus Heat Shock-IGF group, respectively. In conclusion, IGF-I exerted a thermoprotective effect reducing heat-induced apoptosis in bovine oocytes.

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Protocol for ovulation induction in prepubertal heifers

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Introduction

Recently, several hormonal protocols were utilized for ovulation induction in prepubertal heifers, decreasing age at first calving and increasing reproductive efficiency. For non-ovulating heifers the first procedure is to expose the animal to progesterone, that modifies estradiol excessive negative feedback, reorganizing the hypothalamic-pituitary-gonadal axis (1). After progesterone device removal heifers can either be stimulated with GnRH or its analogs for ovulation (2). Progesterone excess can jeopardize synchronization protocol in prepubertal heifers and there is no specific progesterone device to be used in this animal category (2). The present research compared the efficiency of buserelin (0.2 mg) and p-LH (25 mg) for ovulation induction in follicles larger than 8mm, from prepubertal Nelore heifers previously submitted to a hormonal protocol for puberty induction.

Material and Methods

Prepubertal Nelore heifers (n = 24) with 21 mo of age, weighing 334 kg (381 to 291 kg) were used. All heifers were submitted to the same synchronization protocol: on day 0 an used (4^{th} time) intravaginal progesterone device (Sincrogest®, 1g-1^{st}use) was inserted, and heifers were injected with 2mg IM of estradiol benzoate (Sincrodiol®); after progesterone device removal 50 mg of cloprostenol IM (Sincroforte®) and 1mg of estradiol were injected. After 48 hours animals were injected either with 0.2 mg, I.M. of buserelin (Sincroforte®,) or with 25 mg, I.M. of pLH (Lutropin®). Daily ultrasound examination was performed during the experimental period and every 6 h for 24 h after ovulation induction injection to measure follicle diameter and detect ovulation.

Results and Discussion

From 24 heifers, 8 were excluded due to small follicle diameter (<6mm) at intravaginal progesterone device removal. Progesterone associated to estradiol decreased largest follicle diameter from 10 ± 1.75mm to 6.69 ± 0.95 mm on day 5. Both ovulatory inductors induced the same ovulation rate 62.5% (5 out of 8), there was no difference (p = 0.76) on pre-ovulatory follicle diameter between GnRH (8.63 ± 1.06mm) and LH (8.5 ± 1.93mm) treated animals. Growing follicles were able to ovulate when submitted to an increased LH concentration. As those heifers were close to puberty, considering weight (381 kg) and age (21 mo) they have enough LH stored to induced ovulation after GnRH stimulation (3, 4). Exogenous progesterone exposure associated with estradiol, followed by either LH or GnRH stimulation was effective to induce the first ovulation in prepubertal Nelore heifers.

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Polymorphism of the BMP-15 gene in Santa Inês and Morada Nova ewes

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Introduction

The discovery of gene mutations related to prolificity in ovine breeds and lineages has helped to drive genetic improvement programs in herds. The bone morphogenetic protein 15 (BMP-15) gene is linked to the X chromosome and was denominated FecX (1). The *Fec*X^{G} mutation (Galway - Q239Ter) is characterized by an exchange of nucleotides that results in the insertion of a premature stop codon in the transcription of the mRNA and that, in heterozygosis, has been associated with an increase in the ovulation rate of Cambridge and Belclare sheep (2). The other mutation point *Fec*X^{I} (Inverdale – V31N) was first identified in Romney sheep (3) and results in the VAL – ASP change.,This mutation in heterozygosis also increase ovulation rate. Both mutations result in sterility when in homozygosis. The Santa Inês breed has considerable influence in the formation of commercial sheep herds in Brazil. Among those that formed the Santa Inês breed, the Morada Nova breed is cited as the most prolific, with its presence owing to a few persistent sheep breeders. Thus, the aim of the present study was to detect the polymorphism of a single nucleotide from the BMP-15 gene (*Fec*X^{G} and *Fec*X^{I}) of the Santa Inês and Morada Nova breeds.

Materials and Methods

Ewes were selected (100 Santa Inês and 80 Morada Nova) from the semi-arid region of northeastern Brazil. All ewes had a history of at least three parturitions, with single, 2 or 3 multiple-offspring births. The genomic DNA was extracted from leukocytes using the modified phenol-chloroform protocol. DNA fragments were amplified by polymerase chain reaction (PCR), using the following oligonucleotides: *Fec*X^{G} – F5' CACTGTCTTCTTGTTACTGTATTTCAATGAGAC and R5' GATGCAATACTGCCTGCTTG – *Fec*X^{I} - F5' GAAGTAACCAGTGTTCCCTCCACCCTTTTCT and R5' CATGATTGGGAGAATTGAGACC. These oligonucleotides insert a mutation point in the amplified fragment, which generates a forced restriction site for the *Hinf* I (*Fec*X^{G}) or *Xba*I (*Fec*X^{I}) enzymes, in the restriction fragment length polymorphism technique (RFLP). The digested DNA was observed by electrophoresis, and the data on the bands found in each group of sheep were analyzed with regard to frequency and related to the prolificity phenotype.

Results and Discussion

For the Morada Nova breed, 40% of the herd presented a history of at least one multiple-offspring birth, whereas the Santa Inês breed showed 25% of multiple-offspring births. The prolificity index was 2.39 and 2.13 for the Santa Inês and Morada Nova breeds, respectively. No mutations referring the *Fec*X^{G} and *Fec*X^{I} gene were found in both breeds. PCR-RFLP revealed that all wild-type bands were cut by the *Hinf* I enzyme (G/ACT), resulting in fragments of 111pb and 30pb, which characterized an absence of the *Fec*X^{G} gene mutation. On the other hand, the *Fec*X^{I} fragments of 154pb weren't cut, therefore, no restriction site for the *Xba*I enzyme was observed. As no *Fec*X^{G} and *Fec*X^{I} mutations related to the BMP15 gene were found in the present study, we assume that no correlation with prolificity can be inferred. Hence, the use of surveys related to these mutations as a marker of prolificity is not an adequate tool for commercial improvement programs for this trait **in** Santa Inês and Morada Nova ewes. However, it should be considered that the studies on phylogeny in sheep are based on the investigation of common polymorphisms between breeds or lineages. For example, the Booroola gene has been chosen for studies on binding maps. Thus, such studies on ovine brazilian breeds with a history of prolificity are important.

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Bovine dominant follicular fluid promotes the *in vitro* development of caprine preantral follicles

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Introduction

The supplementation of *in vitro* maturation medium with follicular fluid (FF) from large follicles, instead of serum, was reported to enhanced developmental competence of oocytes and growth of preantral follicle in pigs (1, 2). No studies have been related the use of follicular fluid on *in vitro* culture of preantral follicles in goats. Thus, the aim of this study was to evaluate the effect of follicular fluid originated only from cattle dominant follicles on the *in vitro* development of caprine preantral follicles.

Material and Methods

Isolated preantral follicles from 40 caprine ovaries obtained at a local slaughterhouse were pooled and selected for culture. The bovine follicular fluid (bFF), was obtained from the largest follicle present in each ovary from eighteen cows previously synchronized by ablation for follicular ovum pick-up. Isolated preantral follicles were randomly distributed in four different treatments: MEM^+ (control) or supplemented with 10% bFF added on day 0 (D0) until day 18 (D18) (bFF0-18), added on day 6 (D6) (bFF6-18) and added just on day 12 (D12) (bFF12-18). The follicles were disposed in group of three follicles per 100 µL microdroplets of cultured medium covered with mineral oil at 39°C, 5% CO_2 in air and every alternate day medium was replaced partially.

Results and Discussion

The main results of viability, antrum formation and growth rate are on table 1. It was found that the addition of follicular fluid in the beginning of culture promotes the viability, follicular growth and early antrum formation. This work is an agreement with Metoki et al. (2) who demonstrated the supplementation of the culture medium with FF improves the growth of preantral follicles. Numerous compounds present in dominant follicular fluid (3) have the ability to promote the cell proliferation and oocyte development thus, enhancing the follicular growth (2, 4). In conclusion, bovine follicular fluid originating from dominant follicles can maintain the viability and promotes the *in vitro* growth of goat preantral follicles.

Table 1. Viability and growth of caprine preantral follicles cultured with bovine follicular fluid (bFF) added on different periods of culture.

	Viability D18 (%)	Antrum D6 (%)	Diameter D18 (µm)	Growth rate µm/day
ControlMEM^+	38.33%^{B}	53.33%^{B}	478.95^C	13.07^B
bFF0-18	66.67%^{A}	76.81%^{A}	557.56^B	16.61^A
bFF6-18	52.00%^{AB}	53.33%^{B}	762.00^A	22.52^A
bFF12-18	38.71%^{B}	56.45%^{B}	603.53^C	12.18^B

Differents superscripts (^{A,B}) between rows differs significantly.

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A novel protocol achieving synchronized ovulations in Santa Inês ewes

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Introduction

Traditional treatments for estrous synchronization in ewes were developed several years ago. To achieve god results this protocols depend on oestrus or ovulation detection before natural breeding or artificial insemination (1, 2). Besides in Santa Inês breed there is a lack of information regarding the efficiency of those protocols. The aim of the present study was to investigate the effect of human chorionic gonadotrophin (hCG) injection on a synchronization protocol in Santa Inês ewes.

Material and Methods

The experimental design, cross over was conducted in two consecutive steps. Nine ewes were randomly divided in one of two treatments: Control Group (CG) and hCG Group (hCGG). All the ewes received an intravaginal progesterone device (Primer-PR®, Tecnopec, Brasil) on D0 which were removed on D9. Also on D9 they received 100 µg of d-cloprostenol (Prolise®, Syntex, Argentina). Animals on CG also received 250 UI of eCG (Folligon®, Intervet, Holanda). On D10 the ewes in the hCGG, received 500 UI of hCG (Vetecor® - Hertape Calier-Espanha). Ultrasound examinations (Aloka SSD-500) were performed every 12 h from D9 until ovulation. Moreover, oestrous detection was performed three times a day (10, 18 and 24 h) with an vasectomy male. Statistical analysis was performed by GLM and GLIMMIX procedures of the Statistical Analyses System (SAS). Continuous data were tested for normality of residuals and homogeneity of variances and transformed when necessary.

Results and Discussion

Higher estrus behavior was found in the hCGG ($p < 0.01$) than CG. Shorter interval device removal/ ovulation ($p < 0.01$) was noticed. Besides, ovulation occurred more synchronized in the hCGG. No significant difference between experimental groups was noticed in the interval device removal/standing heat ($p > 0.5$).

Table 1. Effect of hCG injection on a synchronization protocol in Santa Inês ewes.

	CONTROL	hCG	*p*
Oestrus behavior (%)	100 (9/9)	37.5 (3/8)	0.009
Interval device removal/standing heat (h)	47.4 ± 15.0	32.0 ± 0.0	0.11
Interval device removal/ ovulation (h)	79.9 ± 15.4	54.7 ±4.9	0.001

These results lead to the conclusion that hCG administration in Santa Inês ewes eliminate the need of oestrous detection. This hCG protocol achieve higher synchronization of ovulation with beneficial practical implications specially to perform artificial insemination.

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LH secretion in prepubertal Nellore heifers after lecirelin injection

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Introduction

GnRH analogs alter follicular dynamics and synchronize estrus in cattle (1). Upon induction of a preovulatory LH surge, GnRH causes ovulation of a dominant follicle and emergence of a new follicular wave within 2 or 3 days after injection (2). Apparently, LH secretion is restrained in prepubertal heifers due to a suppressed GnRH secretion, however, it is not known whether the pituitary has LH stored in the gonadotropes that may be released in response to an injection of GnRH. Objective was to evaluate LH secretion after a GnRH (Lecirelin 0.25 mg) injection in prepubertal Nellore heifers.

Material and Methods

Ovaries of 20 Nellore heifers (average body weight 324 ± 26 kg) were evaluated twice every 24h by ultrasound. Heifers were grouped according to the diameter of the largest follicle (8.5-10 mm and >10 mm) and received an injection of either lecirelin (Gestran®, 0.25 mg/animal I.M; Treated group, n = 10) or saline (4 ml; Control group n = 10). After GnRH injection, ovaries were evaluated by ultrassonography (Prosound 2, transducer 5 Mhz, Aloka®) every 24 h for 3 days to check for signs of ovulation. Blood samples were collected every hour during a 6h period and concentrations of LH were measured by RIA (0.0625 sensitivity, CV intra-assay 5.5 % and inter-assay 9.58%). Data were analyzed by ANOVA.

Results and Discussion

Lecirelin treatment stimulated the secretion of an LH peak 2 h after injection in prepubertal heifers. Treated animals had both a greater peak area ($p = 0.0031$; 16.09 (ng/ml)h) compared to control (saline) heifers (0.68 (ng/ml)h), and a higher peak amplitude (19.12 ng/ml) compared to control (2.50 ng/ml; $p = 0.002$). There was no difference ($p = 0.322$) on the diameter of the largest follicle between treated (10.20 ± 0.92mm) and control groups (9.70 ± 1.25 mm), during the two ultrassonographic periods. Exogenous GnRH stimulated LH secretion from the pituitary that is important for follicle maturation (3) but it did not affect the largest follicle diameter. No heifer ovulated within 48h after GnRH injection. It can be concluded that 0.25 mg of licerelin was effective to induce a LH peak in prepubertal Nellore heifers but there was neither an increase on largest follicle diameter nor ovulation, as expected (4).

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In vitro culture of bovine preantral follicles in the presence of indol acetic acid

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Introduction

In animal cells, "Indol-3-acetic acid (IAA)" has been used to preserve goat preantral follicles (1) and acts as an antioxidant by inhibiting peroxidation (2), which may be important for follicular survival *in vitro*. However, the effects of IAA on *in vitro* cultures of bovine ovarian preantral follicles remain unknown. The objective of this study was to evaluate the development of preantral follicles from the Nelore breed of *Bos indicus* after *in vitro* culturing of ovarian cortices in different concentrations of IAA. In addition, this study investigated the possible association of mammalian genes with IAA activity in bovine preantral follicles using an in silico approach.

Materials and Methods

Ovaries (n = 8) from Nelore heifers were collected, and the ovarian cortex was divided into 14 fragments. Of these samples, two fragments were immediately fixed in Carnoy solution (control), and the other fragments were cultured individually at 39°C with 5% CO2 for two or six d in Minimum Essential Medium (Sigma, St Louis, MO, USA) supplemented (MEM+) with penicillin, streptomycin, BSA, insulin, transferring, selenium, pyruvate, glutamine and hypoxanthine. This MEM+ was supplemented with different concentrations of IAA (10, 40, 100, 500 or 1000 ng/ml). After being cultured for either 2 or 6 d in each media, pieces of the ovarian cortex were fixed in Carnoy solution. After fixation, tissues were mounted on histology glass slides and stained using a standard hematoxylin-eosin protocol. The preantral follicles were classified according to the stage of development. In total, 120 follicles were studied per culture medium and per culture duration. When evaluating the rates of follicle activation and growth, only intact follicles were considered, and the percentage of primordial and developing follicles was calculated on Day 0 (control) and after 2 or 6 d of culture in each treatment. The functions of the selected mammalian genes were verified by SWISS-PROT (3), and the metabolic pathways in which these gene products were involved were defined based on the "Kyoto Encyclopedia of Genes and Genomes (KEGG)". The selected sequences from *Arabidopsis thaliana, Bos taurus* and *Ovis aries* were aligned using the ClustalW program. Pair-wise comparisons were conducted using Tukey's ($P < 0.05$) with the Statistical Analysis System (SAS Institute, Cary, NC, USA).

Results and Discussion

Compared to the control culture, the percentage of primordial follicles was reduced ($P < 0.05$) and the percentage of developing follicles was increased ($P < 0.05$) after 2 or 6 d of culture in all media tested. Furthermore, culturing of the ovarian cortex for 6 d reduced the percentage of healthy, viable follicles when compared with the control ($P < 0.05$). In contrast, cultures supplemented with 10 ng/ml of IAA were the only samples that had similar ($P > 0.05$) percentages relative to the control group. In *in vitro* cultures of sheep ovarian preantral follicles, a concentration of 40 ng/ml of IAA has been shown to induce follicular activation (4). Finally, we found a mammalian gene that was homologous to the plant gene, ROOTY, which may be involved in the oocyte response to IAA. In conclusion, Nelore bovine primordial follicles may be successfully activated *in vitro* after culturing for 6 d in MEM containing 10 ng/ml of IAA.

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Plasma and urine progesterone concentration in nellore heifers

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Introduction

Alternative endocrine monitoring, that does not include physical contention for successive blood sampling, diminishes stress and interference with reproductive hormones (1). Non-invasive methods can be used to evaluate variations on the concentration of individual steroid hormones in different periods and environment situations (2). Concentrations of progesterone metabolites have a positive correlation with CL number and early progesterone production from placenta (3).This study evaluated progesterone concentrations from heifer's blood and urine samples, exploring new sampling ways for hormone analysis in cattle.

Material and Methods

Blood samples (10 ml) were collected every hour from 7:00 to 19:00 from the jugular vein and from spontaneously urinated samples (approximately 10 ml) from three Nellore heifers (*Bos taurus indicus*), yielding 13 blood and urine samples per animal. Blood samples were mixed with 200µl of an anticoagulant solution (EDTA) and centrifuged at 1500 rpm for 15 min to obtain plasma. Serum and urine progesterone concentration were quantified by radioimmunoassay using the DPC Coat-a-Count kit (CV 0.15%). Wilcoxon matched-pairs signed-ranks test and Non-parametric Spearman correlation (GraphPad, Instat) were used for data analysis.

Results and Discussion

Plasma (4.43 ± 0.25 ng/ml) and urine (4.01 ± 0.52 ng/ml) progesterone concentrations did not differ ($p = 0.1737$). Associated with the lack of difference there was ($p = 0.0009$) a positive correlation ($r = 0.4824$) between plasma and urinary progesterone concentration, as described previously (3). In summary, steroid hormone quantification from bovine urine was possible; urine progesterone concentration did not differ from plasma concentration, suggesting that renal steroid excretion closely resembles blood concentration at that moment. Despite the positive correlation reported here, there seems to be a delay of nearly two hours for increases in blood hormone concentrations to be detected in the urine, that needs further evaluation.

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