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Comparison of objective and subjective methods for evaluating the vascular pattern of the preovulatory follicle using color Doppler

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Keywords: color doppler, follicle, vascularization.

Different studies have demonstrated the influence of follicular blood flow in the quality and developmental potential of oocytes. The ultrasonography Color Doppler mode allows to identify and to measure the vascular pattern associated with ovarian structures, and is receiving increasing attention as a tool for research and diagnostics. The vascularization measurement, however, generally requires the capture and processing of images, limiting its use in a large number of animals. The purpose of this study was to evaluate the agreement between the measurement (objective) and visual assessment (subjective) in determining the vascular pattern of the wall of ovarian follicles. Were used 60 crossbred Holstein-Zebu, subject to standard protocol for synchronization of follicular growth: Day -7, insertion of intravaginal (1.0 g progesterone, Sincrogest, Ourofino, Brazil) and application of 2 mg im of EB (Sincrodiol, Ourofino); D0, implant removal and application of 0.5 mg cloprostenol (Sincrocio, Ourofino). The pre-ovulatory follicles were identified and evaluated by Color Doppler (MyLab30 VetGold, Esaote, Italy) using a linear rectal tranducer with 7.5 MHz, color gain of 70% and 0.7 KHz PRF. The same frequency and pattern of gains and color were used in all evaluations. A sequence of images of each follicle was recorded for later analysis. The objective evaluation was calculated by the ratio of the vascularization area and the total area of the follicle wall, the image with maximum Doppler signal, measured by a function of the equipment. For subjective evaluation was created a visual scoring scale of 1-4, corresponding to vascularization, intense, good, average and low, respectively. We evaluated 69 pre-ovulatory follicles, dominant or co-dominant, with a medium diameter of 12.0 ± 2.7 mm. The average area of the follicular wall was 0.46 ± 0.17 cm^2 and 0.22 ± 0.10 cm^2 of vascularization area. After the distribution into quartiles, based on the vasculature, the percentage of follicles in each quartile did not differ between the subjective and objective evaluation (43.5%, 34.8%, 17.4% and 4.3% vs. 30, 4%, 44.9%, 20.2% and 4.3%, for the 1st, 2nd, 3rd and 4th quartiles and scores 1, 2, 3 and 4, respectively). The result of the two forms of assessment showed a strong association (Goodman and Kruskal's Gamma = 0.85, $p < 0.001$), and only one follicle was evaluated over a class mismatch between the two techniques. In conclusion, the subjective evaluation technique is effective for evaluation of follicular wall vascularization in cattle follicle, and can be used in situations where the need for decision-making limits the processing of subsequent images.

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Effects of the association of glycerol and ethylene glycol on cryopreservation of ovine sperm

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Keywords: freeze, membrane,sperm.

Glycerol is the most widely used cryoprotectant in the freezing of semen in ruminants, but has toxic effects on sperm. Alternatively it has emphasized the use of ethylene glycol due to its ability extender, lower molecular weight and easier to cross the plasma membrane. The aim of this study was to compare the effect of the combination of ethylene glycol and glycerol as cryoprotectants for freezing ram semen, analyzing their effect on: total motility (MT), progressive motility (MP), vigor (V) and membrane functionality (hypoosmotic test - HOST) after thawing. Seventeen ejaculates collected with an artificial vagina were analyzed. Seminal parameters were measured before and after freezing. The cooling of semen was performed in two stages, in Styrofoam box. Firstly, semen was diluted in fraction A (FA: TRIS-yolk), and incubated for 1 hour until 5°C where it remained a further 1 h. Next, was added fraction B (FA + cryoprotectant) (1:1) containing 14% glycerol, 6% ethylene glycol (control groups) or glycerol (3.5%) + ethylene glycol (1.5%) where they remained for 30 minutes at 5°C. Semen was packaged in 0.25 ml straws, and placed 6 cm from the level of liquid nitrogen (N2L) for 15 min. Straws were dipped in N2L and stored in cryogenic container. The straws were thawed in a water bath at 37°C. The evaluation by HOST, 200 cells were counted of each palette, where cells with curly tail were identified as intact cells and cells of tail straight were rated as damaged. The data were subjected to analysis of variance and means were compared by t-test with 5% of probability. The results showed significant difference on the physical parameters between samples frozen with glycerol (MT=48.52±18.26; MP=34.11±8.52 and V=3.47±0.87) and ethylene glycol (MT=37.05±18.54; MP=27.94±13.58 and V=3.1±0.78). However, the frozen samples with the combination of glycerol and ethylene glycol did not differ significantly from those frozen with glycerol or ethylene glycol (MT=47.35±15.52; MP=36.17±10.68 and V=3.76±0 56). The evaluation of sperm by HOST showed no significant difference between the frozen samples in the presence of glycerol, ethylene glycol or the combination of glycerol and ethylene glycol (66.41%±21.68; 69.79%±14.90; 67.29%±15.68, respectively). Considering the various treatments had similar results by the HOST, and based on analyzes of the physical parameters measured, the combination of glycerol and ethylene glycol cryoprotectants may be used as diluents component for cryopreservation of semen sheep, the same as glycerol. Therefore, it is important to evaluate other semen parameters, as well as the pregnancy rate to obtain more precise information about the possible benefits of the association of cryoprotectants.

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Levels of mRNA for BMPRIB, BMPRII and FSH-r in bovine ovarian follicles grown *in vivo* (0.3mm) and *in vitro* cultured

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Keywords: bovine, mRNA, ovarian follicle.

The objective of this study was to investigate the expression of BMP-15 receptors (BMPR-IB and BMPR-II) and FSH receptor (FSH-R) in bovine follicles grown in vivo (~0.3 mm) and cultured in vitro for 12 days. Follicles of approximately ~0.2 mm (selected to in vitro culture) and ~0.3 mm of diameter (stored -80°C at extraction of RNA) were identified and manually microdissected. After selection, follicles ~0.2 mm were individually cultured for 12 days at 39°C and 5% CO_2 in air, in α-MEM + (cultured control), BMP-15 (D0 – D12: 50 ng/mL), sequential rFSH (D0 – D6: 50 ng/mL and D7 – D12: 100 ng/mL) or sequential BMP-15 + rFSH (D0 – D12). For both categories of follicles (grown in vivo and in vitro), total RNA extraction was performed using Trizol® (Invitrogen, São Paulo, Brazil), followed by the reverse transcription reaction. mRNA quantification was performed using SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA), using specific primers for BMPRIB, BMPRII and FSH-R. The housekeeping gene used to normalize the levels of mRNA was ubiquitin (UBQ). Data of mRNA expression are given as the mean ± standard deviation (SD). ANOVA using General Linear Model (GLM) procedure of SAS was used to test the effect of BMP-15 and FSH on the relative expression for BMPR-IB, BMPR-II and FSH-R on fresh control and follicles growth in vitro for 12 days in different treatments, following by Tukey test to locate differences among treatments ($P<0.05$). The results showed follicles growth in vitro in α-MEM + and BMP-15, respectively showed a significant increase in the mRNA level for FSH-R (6.43±1.44; 18.52±6.48) when compared with follicles growth in vivo (2.30±0.97; 2.89±1.63) ($P<0.05$). In contrast, in vitro culture in BMP-15 reduced the mRNA level for BMPRII (1.20±0.09) ($P<0.05$) when compared with follicles with 0.3 mm (4.46± 1.37). In follicles cultured in BMP-15 + FSH (D0-D12): 1.67±0.31 and BMP-15+FSH (D7-D12): 1.16±0.10, the mRNA levels for BMPRIB had significantly declined compared with follicles grown in vivo in BMP-15+FSH (D0-D12): 5.20±1.34; BMP-15+FSH (D7-D12): 3.32±0.42 ($P<0.05$). In conclusion, the expression of BMP (BMPR-IB, and BMPR-II) and FSH receptor genes was altered by in vitro culture.

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Effect of cryopreservation of *in vitro* produced bovine embryos on membrane lipids profile by mass spectrometry

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Keywords: cryopreservation, embryo, mass spectrometry.

This study aimed to evaluate changes in membrane lipid profile obtained by mass spectrometry (MS) after cryopreservation of *in vitro* produced (IVP) bovine embryos. COCs were *in vitro* matured (IVM) for 24h, and after IVF, zygotes were IVC in SOFaa medium (5 mg/mL BSA + 2.5% FCS, 5% CO_2 in air) for 7 days, when blastocysts were vitrified (Ingámed®, Maringá-PR, Brazil). Then, they were heat to evaluate embryonic survival. The viable embryos after vitrification, and fresh blastocysts were transferred to microtubes containing 200 µL of methanol HPLC 50% in aqueous solution, stored at -20°C and immediately transported for MS analysis. For the MS analyzes by matrix-assisted laser desorption and ionization (MALDI), each embryo was deposited at the center of the spot's plate. Before analysis, 1 µL of matrix (1.0 mol/12.5 dihydroxybenzoic acid (DHB) in methanol) was deposited on each spot, at room temperature until its complete crystallization. The spectra data were obtained in the range of 700-1200 *m/z*, in positive and reflectron modes with the mass spectrometer Autoflex III (Bruker Daltonics, USA). After excluding isotope peaks, the 50 most intense ions of each spectrum were considered as starting point for determining the *m/z* ratio corresponding to lipids. Only *m/z* clearly distinguished from noise in the spectra were included in the PCA analysis. Due to conditions established for analysis, was expected to detect mainly the membrane lipids, such as phosphatidylcholines (PC) and sphingomyelins (SM). The values of *m/z* which had increased intensity (2 to 10x) on vitrified group and in its probable assignments were: 703 $[SM\ (16:0)+H]^+$; 722 [PEp (36:5)]; 758 $[PC\ (34:2)+H]^+$; 762 $[PC\ (34:0) + H]^+$; 790 [PC (36:0)] and 811 $[PC\ (40:6)+K^+$ loss of $N(CH_3)_3]$. The m/z ion 744 [PCp (34:1) or PCe (34:2)] was 3.7 times more intense on fresh group. These assignments were based on previously published works. Interestingly, ions of 722 and 744 *m/z* indicate the presence of lipid species containing fatty acid residues linked through ether type bonds (alkyl ether or plasmalogens, indicated by the lowercase "e" and "p", respectively) to the glycerol structure, which are more resistant lipids to enzymatic degradation and commonly found in sperm. This observation must be confirmed by fragmentation experiments. These results indicate that cryopreservation significantly alter the membrane lipid profile. These alterations can be monitored by MS and may reflect the metabolic response to injury or change in environmental conditions.

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Effect of different cryopreservation protocols on re-expansion and hatching rates of *in vivo* produced sheep embryos

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Keywords: cryopreservation, embryo, ovine.

Currently, slow freezing and vitrification are the two available techniques for cryopreservation of sheep embryos. The aim of this study was to evaluate the effects of slow freezing and vitrification by cryotop, on re-expansion and hatching rates, after thawing and warming. Thirty-two multiparous ewes from Santa Ines breed, submitted to a superstimulatory protocol were used to perform the experiment. An intravaginal sponge containing 60 mg of medroxy acetate progesterone (MAP, Progespon®) was used during 14 days. Seven days after the insertion, the sponge was changed and an application of 0.5 ml of PGF2a (Veteglan®) IM was performed. On Day 12 the superstimulatory treatment was initiated using a total of 200 mg FSH (Folltropin®) administrated IM twice daily in decreasing doses over a 4-day period. On Day 14 the sponge was removed and the animals received 200 IU of eCG (Novormon®) IM. Insemination was performed on day 16, by laparoscopic technique, using fresh semen at 36 hours after MAP removal. Embryos were recovered on D5 after insemination by laparotomy. Only grade I and II compact morula were used, and were divided into three groups: non-cryopreserved control group (CG, n = 31), slow freeze (CON, n = 31) and vitrified (VIT, n = 28). During cryopreservation process, embryos from GC group were kept in LAV medium (TCM-199 Hank's, Gibco BRL, Burlington, ON, Canada) supplemented with 10% FCS on the bench. After warming of CON and Vit groups, the three groups were transferred to SOF medium and cultured at 39°C in 5% CO_2 in air. Embryos were evaluated for re-expansion and hatching rates at 24, 48 and 72 hours of culture. Using Qui-Square test data were cosidered diferent when $P<0.05$. There were no differences ($P>0.05$) in re-expansion and hatching rates between CON and VIT groups at 24 (65%; 10% and 20%; 0%), 48 (58%; 35% and 57%; 25%) and 72 hours (58%; 52% and 57%; 43%), respectively. CG had the highest ($P<0.05$) re-expansion rates at 24 (94%) and 48 hours (87%), but with 72 hours (81%) was similar ($P>0.05$) to cryopreserved groups. Hatching rates of CG were similar ($P>0.05$) to cryopreserved groups at 24 (0%) and 48 (32%). Also at 72 hours CG (74%) showed similar ($P>0.05$) rates to the CON. However, when compared to VIT, CG presented higher ($P<0.05$) rate of hatched embryos. The results suggest that both cryopreservation protocols are effective methods for cryopreservation of ovine embryos. However, more studies are needed to elucidate which technique is best to store this type of germplasm.

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The use of real-time ultrasonography as tool for selection of oocyte donors in IVP goat programme

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Keywords: goat, ovary, ultrasound.

In goats, a major limitation to the application of in vitro embryo production (IVP) is the occurrence of a large variability in the stimulatory response in goats, which is unable to estimate the ovarian response in these animals when treated hormonally, according to (MENCHACA et al., 2002, Theriogenology, 58: 1713-1721). The objective of this study was to determine the ultrasound efficiency to estimate an appropriate minimum response for donor inclusion in a laparoscopic oocyte recovery. This study was performed using 15 Canindé goats. Goats were treated with intravaginal sponges containing 60 mg MAP (Progespon, Buenos Aires, Argentina) for 10 days. A single i.m. injection of 75 µg d-cloprostenol (Prolise, São Paulo, Brazil) was administered on the 8th day of progestagen treatment. The animals were treated with a total of 120 mg NIH-FSH P1 (Folltropin-V, Belleville, Canadá) divided into five doses at 12-h intervals and started 72 h prior to sponge removal. Transrectal ultrasonographic examination of ovaries was performed, in all goats, using a 6.0/8.0 MHz linear-array probe, fitted to a Falco 100 B-mode scanner (Pie-Medical, Maastricht, Netherlands), just prior to laparoscopic procedure. Each ovary was scanned in different planes to ensure that all follicles ≥2mm were seen. To confirm the ultrasound examinations, females were submitted to laparoscopic procedure, in which an endoscope was used (Karl Storz, Tuttlingen, Germany). In laparoscopy, ovarian response was classified into two types: <10 or ≥10 viewed follicles per animal. The procedure is feasible to harvest oocytes only in animals with superior response to 10 follicles. These procedures were performed in double blind. The results were analyzed using the Statistica 7.0 and the values expressed as mean±SD. Differences between means were analysed by χ^2 test, $P < 0.05$. By ultrasound was found that 100% of the animals showed an ovarian response higher than 10 follicles. These results were confirmed by laparoscopy, thus validating the accuracy of ultrasound technique. A total of 261 and 270 follicles were visualized by ultrasonography and laparoscopy, respectively. The stimulatory response observed was 17.4±3.1 and 18.0±5.4 by ultrasonography and laparoscopy, respectively. No significant difference was found between methods evaluated for the type of ovarian response and mean number of follicles visualized ($P > 0.05$). Therefore, it was concluded that ultrasonography is an efficient technique to select donor oocytes with sufficient response to be included in IVP program in goats.

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Viability of equine mesenchymal stem cells subjected to different cryopreservation protocols

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Keywords: cryopreservation, stem cells, viability.

Using two different protocols for cryopreservation we compared the cell viability of Mesenchymal Stem Cells from bone marrow. Methods: Both protocols were based on DMSO. The first protocol was performed with high glucose DMEM (35%), F12 (35%), bovine fetal serum (20%), antimycotic (1.2%), antibiotics (1%) and DMSO (10%). The second protocol was used Fetal Bovine Serum (90%), DMSO (10%), antimycotics (1.2%) and Anti-Bacterial (1%). For each protocol, three samples, in duplicate, were used. Results: The cell viability test was made by the method of counting using a Neubauer chamber with Trypan Blue vital stain. In the first protocol after freezing, the mean viability was 100,500 cells/ml to 350,000 cells/mL, and its pre-freezing viability was 730,000 cells/ml to 815,000 cells/mL, obtaining a viability of approximately 43%. For the second protocol viability pre-freezing was 930,000 cells/mL by the 1,330,000 cells/ml, and after freezing viability was 630,000 cells/ml to 810,000 cells/mL, and the viability of approximately 61%. Conclusion: It was noted that the use of fetal bovine serum in conjunction with DMSO without adjuvants, promoted greater protection and preservation of cellular structure, thus increasing the index of cell viability.

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Neuropeptide y-containing axons are located in close proximity to kisspeptin neurons in the hypothalamus of ewes

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Keywords: cyclicity, neuroendocrinology, reproduction.

The modulatory role of nutrition on reproductive function involves, at least in part, the action of Neuropeptide Y (NPY) on regulating the release of gonadotropin-releasing hormone (GnRH). The objective of this study was to investigate the presence of functional structures that could indicate a role for kisspeptin (KP) neurons as mediators of NPY actions on the reproductive axis. Specifically, close contacts between NPY-containing axons and neuronal cell bodies and dendrites immunoreactive to KP were determined in the pre-optic (POA)/periventricular (PeV) areas and arcuate nucleus (ARC) of the hypothalamus. Adult, Suffolk-crossbred ewes (n=3) were ovariectomized and an implant containing estradiol 17-beta was inserted subcutaneously to maintain the circulating concentrations of estradiol similar to those observed during the early follicular phase. One month after the ovariectomy and during the breeding season, ewes were euthanized and a block of tissue containing the hypothalamus was collected. Tissue was sectioned (50 μM) and processed for detection of KP and NPY by dual-label immunofluorescence. Tissue sections 200 μM apart were analyzed by microscopy. The number of NPY-containing varicosities in close proximity to KP cell bodies and dendrites, and the density of NPY-containing fibers in various hypothalamic areas were determined. Consistent to the use of estradiol implants in this experimental model, mean concentrations of estradiol in serum, determined by radioimmunoassay, averaged 3.7±0.7 pg/mL. Similar to other studies, KP neurons were observed to concentrate in the POA/PeV and ARC. In the POA/PeV, it was observed on average 89±75 KP neurons, of which approximately 10% were in close proximity to NPY-containing fibers. In the ARC, it was observed on average 795±108 KP neurons, of which 30% were in close proximity to NPY-containing fibers. The mean number of NPY varicosities in close proximity to KP neuron cell bodies and dendrites were 1.6±0.3 in the POA/Pev, and 2.1±0.3 in the ARC. Hypothalamic areas with greater density of NPY fibers were the ARC and the median eminence (ME). Additionally, KP neurons in the vicinity of the ME appear to have increased number of close-contacts with NPY fibers. In conclusion, the present study indicates that NPY-containing axons are in close proximity to KP cell bodies and dendrites in the POA/PeV and ARC of ewes. This structural observation indicates that KP neurons may be regulated by NPY and participate in the metabolic control on the reproductive function in sheep.

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Gene expression of luteinizing hormone receptor from two-cells to blastocyst stages mouse embryos

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Keywords: gene expression, LH receptor, mouse embryo.

There is no agreement regarding gene expression of the LH receptor (LHR) in bovine oocytes and embryos, exons where primers should be annealing and sort of PCR (conventional or nested). Taking the mouse as animal model – because preimplantation mouse embryos express LHR – could be used to validate sensitivity, developmental stage and primers annealing site. The aim of this study was to validate LHR gene expression in mouse embryos, with respect to the variables above mentioned, by amplification a fragment containing part of exon 1 and 2, a little studied region that doesn't undergo alternative splicing in rats. SwissWebster females were superovulated according to Manciniet al. (2008.Transgenic Research, 17:1015). Embryos were harvested at 2 cells, 4-8 cells, morula and blastocyst stages and, only those with excellent quality the zona pellucidae was partially removed by Tyrode's acid solution (Sigma). Pools of embryos (n=15/stage) were stored in stabilization solution (RNAlater, Ambion) at -20°C. Ovarian macerate was used as positive control for expression of the LHR and it was stored in the same way. Total RNA extraction was performed according to specifications of manufacturers for each pool of embryos (NucleoSpinXS, Macherey-Nagel) and ovarian tissue (TRIZOL Reagent, Invitrogen). Total RNA from ovarian tissue was treated with DNase I (Invitrogen). All extractions were quantified (260 and 280nm) and 1μg of total RNA was used to reverse transcription (SuperScript III, Invitrogen). cDNA was used for nested PCR with Platinum PCR Supermix (Invitrogen) in a StepOnePlus thermalcycler (Applied). Outer (first amplification) and inner (nested) primers were designed to anneal in exons 1 and 2 of the LHR and to produce a 147bp final amplicon (based on NM_013582.2, GenBank). β-actin was used as housekeeping gene and its primers were obtained from Parakainenet al. (2005.Mol End, 19:2591-602). PCR products were subjected to electrophoresis on 3% agarose gel (100V/3h), stained (GelRed, Biotium) and images digitally captured. Unexpectedly, besides positive control (ovary), only in the morullae stage was observed the in silico expected LHR expression with 147bp. Amplicons with higher (Morullae) or lesser (other stages) molecular weight than 147bp were observed and their identities will be elucidated after sequencing. In silico analysis refused the possibility of dimmers or concatamers of primers, and the higher PCR annealing temperatures (>61ºC) decreases the possibility of unspecific annealing. We conclude that the amplification of exon 1 and 2 didn't enable the embryo mouse LHR expression validation between 2 cells to blastocist, except the Morullae stage and depending of identity confirmation of the amplified product of 147bp.

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Introduction of trehalose by eletroporation to criopreservation of bovine oocytes

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Keywords: electropermeabilization, in vitro maturation, vitrification.

Bovine oocyte cryopreservation is an important procedure used in order to assist reproductives biotechnologies, but still shows unsatisfactory results. The objective of this study was to establish an electroporation protocol with trehalose for crioprotection of bovine oocytes. Three experiments were done in order to establish the better protocol for utilization of the trehalose as intracellular criprotectant. Once the substance isn't natural penetrant in plasme membrane, it is necessary the use of eletroporation for its internalization. Firstly, oocytes in vitro matured were electroporated in plate and cuvette and analyzed at 5 and 30 minutes. As electroporation is characterized by the formation of transient pores, we used the dye propidium iodide (PI) which has characteristic nuclear staining, only penetrating in damaged membranes and there was no statistical difference between the media to percentage of nuclear staining (80±1.82; 10±0.5 and 70±1.0; 15±0.81, respectively), but we preferred to use the plate due to be easer handling. In the second experiment were compared 2 protocols for eletroporation: T1 (4 pulses with 40 V, 2μs), T2 (5 pulses, 130 V, 60μs) and analized with PI and calcein at 5 and 30 minutes after electroporation. At 5 minutes the T1 and T2 oocytes groups showed statistics difference in the staining with PI (75 and 100%, respectively), what didn't ocurred at 30 minutes (15 and 35%, respectively). In the cell viability evaluation, through the staining with calcein, T1 showed larger viability than T2 (84%±0.3 Vs 43.1±0.5) at 30 minutes after eletroporation. In the third experiment, thawed oocyte viability was evaluated, with the groups: G1 (control) - oocytes in vitro matured and vitrified by OPS; G2 - oocytes in vitro matured, eletropored and vitrified by OPS; G3 - oocyte in vitro matured, eletropored and vitrified with reduce the concentration of crioprotectans by OPS; G4 - oocyte in vitro matured, eletropored and vitrified with solution 0.5 M of trehalose by OPS. The oocytes thawed were stained with calcein and there were no statistic differences among groups (G1: 52%; G2: 50%; G3: 53%; G4: 57%) and the low results that can be due the deleterious effects of the cryopreservation process or by eletroporation. In conclusion, the plate is a more indicated support for eletroporation in a bovine oocytes; electroporation is a efficient tecnique to introduce trehalose in a bovine oocytes allowing the permeabilization in a 5 minutes and the restructuring in a 30 minutes; T1 can be used for eletroporation to bovine oocytes; and Trehalose can be used as crioprotectant but more studies are necessaries to permit one better stimate for oocyte viability.

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Oocyte negative pressure treatment increases blastocyst hatching rates

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Keywords: controlled stress, negative pressure, nitrocooler.

The first reports of a positive effect of stress under controlled conditions are from 1899, when after positive hydrostatic pressure followed by a cold shock increased the viability of bacteria found in food. Only in the last decade stress parameters have been optimized for increasing oocyte resistance to procedures such as cryopreservation. Our research group has developed a device that applies different intensities of negative pressure (Nitrocooler). It can produce the necessary controlled stress conditions. The aim of this study was to evaluate the effect of different intensities of negative pressure applied to bovine immature oocytes that were further partenogenetically activated. Ovaries were obtained from an abattoir and oocytes classified as good or excellent were randomly allocated among the groups according to the intensity of negative pressure that was applied during 5 minutes. Group NC 200 – 200 mbar (n = 225); NC 500 – 500 mbar (n = 335); NC 800 – 800 mbar (n = 227); and untreated control (n = 212). Oocytes were activated by 5 minutes exposure to 5 μM of ionomycin followed by 3 h exposure to 2 mM of 6-dymethyl amino purine. Zygotes were cultured in SOFaaci supplemented with 5% of estrous mare serum, in cell culture incubator at 38.5°C, with 5% CO_2 and saturated humidity. Considering day zero (D0) as the activation day, blastocyst (D7) and hatching (D10) rates were used as viability criteria. Data were analyzed by the Chi-square test. Blastocyst rate of NC 200 (25.3%) was significantly lower than control (34.0%) ($P<0.05$), being the latter not statistically different ($P>0.05$) from NC 500 (31.0%) and NC 800 (32.2%). When it comes to hatching rates, NC 500 (40.4%) was higher than control (23.6%) ($P<0.05$), but no statistically different of NC 200 (28.2%) and NC 800 (30.1%) was found. Data show that oocytes submitted to 200 mbar of negative pressure suffer a decrease on embryo development to blastocyst. Also, 200 and 800 mbar of negative pressure do not affect hatching rates. Moreover, 500 mbar of negative pressure enhances embryo hatching rates, evidencing it is possibly the ideal pressure for studies on cryotolerance increase of chemically activated structures.

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Establishment, maintenance and morphological characterization of two novel mammary cell lines from lactating sheep

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Keywords: cell line, mammary gland, ovine.

Mammary gland is a very attractive biological model for study of cellular proliferation, differentiation and organogenesis. However, a few number of mammary gland cell lines were established for farm animals, like ovine. The main objective of this study was to compare two methods, enzymatic and non-enzymatic, to establish ovine mammary cells culture and verify their morphologic characterization. Biopsy of mammary gland (2cm³) was obtained from Santa Ines ewe (90th days of pregnancy). After being minced, the fragments were treated in two groups. The fragments for NDL (no digested line) were in culture medium (DMEM medium - (Gibco®, Grand Island, NY), 10% of FBS (fetal bovine serum), 50µg/ml of gentamicin, 10µl/ml of penicillin and streptomycin solution, 5µl/ml of non essential amino acid and 5µl/ml of Fungizone (Invitrogen®, Grand Island, NY). Tissue for DL (digested line) were smashed across a tea strainer and incubated for 45min at do 37ºC in a digestion media with 0,02% de trypsin, 1% of collagenase A and, 1% of hilauronidase. Both groups were cultured for 7 days at 37ºC and, atmosphere with 5% of CO2 in high humidity. After reach a semi-confluence, the cells were individualized by enzymatic digestion with trypsin 0.01%, plated on new dishes until reach semi-confluence. On order of $1x10^6$ viable cells were cryopreserved. Approximately $3x10^6$ cryopreserved cells for each group were defrosted and plated on 100mm dish for each $1x10^6$ cell. The cells were cultured until passage 7 (P7) and cryopreserved in passage 8 (P8). Same procedure was performed in all passages until passage 12 (P12). NDL and DL had homogeneous growth since P3. NDL in beginning passages have tissue fragments 3 times larger than DL. For passages 0, 1 and 2 cells reached semi-confluence within 15 days, but it was reduced to 10 days from P3 until P5. Time for cell growth was maintained for NDL in 7 days (P5 until P12) during the 8 months of cell culture. However, DL had the same growth time until P10 and after that it was 17 days. The morphologic characteristics were observed in every medium change (48-72 hours) and differences between DL and NDL were noticeable. In NDL from P4 on it was observed two cell morphologies: one rounded with larger surface and another slim with elongations. From P2 to P12 cells clusters were observed in NDL. After at P6 cells were more heterogeneous and in some cell clusters the rounded cells were surrounded by slim cells. Nevertheless, DL always showed highest homogeneity of slim cells, strongly adhered on dish and didn`t have cell clusters after P10. Thus we conclude that the two different methods initially used to obtain the cells line induced the formation of two lines of cells with a different morphology and organization.

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Quantification of mRNA levels for HAS-1, PCNA and Perlecan in bovine ovarian follicles grown *in vivo* (0.2 mm and 0.3mm)

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Keywords: bovine, mRNA, ovarian follicle.

During follicular development, granulosa cell proliferation and antrum formation are both important to increase follicular diameter. Proteoglycans and their glycosaminoglycan side chains are osmotic solutes that act to increase the osmotic pressure inside of the follicle resulting in fluid accumulation (Kovach, 1995, Biophysical Chemistry, 53:181–187; Ishihara et al., 1997, American Journal of Physiology, 272:1499–1506) while Proliferating Cell Nuclear Antigen (PCNA) is expressed in proliferating cells. The aim of this study was quantify the levels of mRNA for Hyaluronan Synthase-1 (HAS-1), Perlecan and PCNA in bovine secondary follicles with 0.2 mm e 0.3 mm grown in vivo. In the laboratory, follicles of approximately 0.2 and 0.3 mm of diameter were manually microdissected and stored at -80° C until extraction of total RNA. Total RNA extraction was performed using Trizol® (Invitrogen, São Paulo, Brazil), followed by the reverse transcription reaction. Quantification of mRNA was performed using SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA), using specific primers for HAS-1 (s: TACTGGGTGGCCTTCAATGT; as: AACTGCTGCAGGAGGTTGTT), Perlecan (s: TGATGAGGCCTCGGGAGACT; as: CGACACCTCTCGGAACTCCT), PCNA(s: TGCCGAGATCTCAGTCACAT; as: TATGGCAACAGCTTCCTCCT) and housekeeping gene Ubiquitin (s: GAAGATGGCCGCACTCTTCTGAT; as: ATCCTGGATCTTGGCCTTCACGTT). ANOVA using General Linear Model (GLM) procedure of SAS was used to test the relative expression of HAS-1, Perlecan and PCNA on the comparison between follicles growth in vivo (0.2mm) and follicles growth in vivo (0.3mm). Data are given as the mean ± standard error (SEM) ($P<0.05$). When the levels of mRNA were compared between in vivo grown follicles (~0.2 and ~0.3mm), the levels of mRNA for PCNA had significantly increased in larger follicles with ~0.3 mm (3.02±0.44) when compared with ~0.2 mm follicles (1.37±0.15) ($P<0.05$). In contrary, larger follicles had lower levels of mRNA for for Perlecan (0.65±0.03) when compared with follicles of ~0.2 mm (2.18±0.63) ($P<0.05$). While the levels of mRNA for HAS-1 did not differ between the follicular categories (~0.2mm: 187.81±199.27; ~0.3mm: 17.15±10.51). In conclusion, for the first time in cattle, it has been found that PCNA levels were higher in follicles ~0.3 mm indicating proliferation in granulosa cells, whereas Perlecan levels were higher in follicles 0.2 mm, suggesting that the action of proteoglycan in the initial formation of antral follicular fluid in bovine.

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Vitrification of imature cumulus-oocyte complexes from *Rattus norvegicus*

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Keywords: oocyte, vitrification, wistar rats.

Viability of rat's oocyte after vitrification is still unsatisfactory. The main supplements used in vitrification solutions are BSA, FCS, PVA and hyaluronic acid. The aim of this work was to assess the rates of meiosis' resumption and in vitro nuclear maturation of Wistar rats' oocytes vitrified with different cryoprotective solutions. Initially, female rats were superovulated with a 20UI eCG injection i.p. and euthanized 48 hours later. After euthanasia, their ovaries were removed from the abdominal cavity and scarified in a plate containing PBS medium to release the cumulus-oocyte complexes (COCs). These COCs were selected under a stereomicroscope and exposed during 4 minutes to a balance solution containing 7,5% ethyleneglycol (EG), 7,5% dimethylsulfoxide (DMSO) and 0,4% BSA. After this time, the COCs were transferred to vitrification solutions which had different compositions depending on the experimental group: G1 - 15% EG, 15% DMSO, 0.5M sucrose, 20% FCS; G2 - 15% EG, 15% DMSO, 0.5M sucrose, 0.4% BSA; G3 -15% EG, 15% DMSO, 0.5M sucrose, 1% hyaluronic acid; and G4 - 15% EG, 15% DMSO, 0.5M sucrose, 0.4% PVA. The G5 was the control group, which was not exposed to the vitrification process. After 1 minute of exposure to the vitrification solutions, the COCs were stored in an open pulled straw (OPS) device and plunged into liquid nitrogen. When the COCs were warmed, they were kept in contact with a solution containing 0.5M sucrose during 5 minutes, then transferred to microdrops containing M16 maturation medium supplemented with 10% FCS, 0.1 IU/mL LH and 5µg/mL FSH. These plates containing the COCs were maintened at 37°C in an incubator with 5% CO2 and 100% relative humidity atmosphere for a period of 27 to 30 hours for maturation. After the maturation period, the oocytes were denuded, transferred to a microdrop containing M2 medium supplemented with 5µg/mL Hoechst and analyzed under a fluorescence microscope. Presence of the first polar body extruded was an indicative of oocyte maturation, and presence of germinal vesicle breakdown was an indicative of meiosis' resumption, as described by Alcoba et al (2012) (DOI:10.1017/S0967199411000463). To compare the rates of meiosis resumption and nuclear maturation between the groups, the Chi-square test was applied supplemented with the calculation of the residues. Differences of $P \leq 0.05$ were considered significant. The meiosis resumption rates obtained in the experiment were 42.3% (11/26) at G1, 21.7% (5/23) at G2, 30% (6/20) at G3, 19% (4/21) at G4 and 80 % (44/55) at G5. The nuclear maturation rates were 38,46% (10/26) at G1, 8,7% (2/23) at G2, 20% (4/20) at G3, 14,3% (3/21) at G4 and 61.8 % (34/55) at G5. Both meiosis resumption and nuclear maturation rates obtained at the control group were statistically higher than the other groups. Groups 1 and 3 presented meiosis resumption and nuclear maturation rates statistically higher than groups 2 and 4. In conclusion, the supplementation of the vitrification medium with 20% FCS or with 1% hyaluronic acid provided higher meiosis resumption and nuclear maturation rates of oocytes from Rattus norvegicus after cryopreservation, although the results are still preliminary.

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Frozen boar semen for fixed-time artificial insemination using different hormonal protocols

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Keywords: cryopreservation, fixed-time AI, semen.

The use of frozen boar semen for artificial insemination (AI) is restricted to the genetic improvement farms. Frozen boar semen is associated with lower conception rates than refrigerated semen. This is mainly due to the short period of sperm viability after the semen thaws in the female genital tract (approximately 4 hours). The use of fixed-time artificial insemination (Fixed-time AI) better synchronizes the time of ovulation and artificial insemination and minimizes the problems associated with short-term viability of frozen sperm. The FTAI protocols commonly used in pigs (with an interval of 72-80 hours between eCG and ovulation inducer) are poorly synchronized, since the amplitude of ovulation is greater than 6 hours (HUHN, Theriogenology, v.46, p.911-24, 1996). An alternative would be to use frozen semen with an interval of 56 hours between hormonal applications. In this case, the amplitude of ovulation would be approximately 3 hours (CANDINI, Brazilian Journal of Veterinary Research and Animal Science, v.41, p.124-130, 2004). Moreover, there is a lack of consensus in the literature as to which ovulation inducer produces better results. This study aims to evaluate the effect of a 56 hours interval between applications of eCG and ovulation inducers (hCG or GnRH) on the fertility of frozen semen. Thirty-two sows in reproduction were divided into three groups. The control group was inseminated 24 and 36 hours after the onset of heat. The hCG treatment group was given 600UI IM eCG at weaning, 500UI IM hCG 56 hours after the eCG, and inseminated 36 and 40 hours later. The GnHR treatment group was given 600UI eCG IM at weaning, 50μg of GnRH IM 56 hours after eCG, and inseminated 36 and 40 hours later. On day 5 after AI, embryos were collected and the fertilization rates (%) and morula (morulas recovered per female) and blastocyst (blastocysts recovered per female) indices reported. Data were analyzed using the chi-square and Fisher´s exact tests (fertilization rate) and one-way ANOVA and Tukey's test (morula and blastocyst rates). There was no significant difference in fertilization rates between experimental groups [84.34% (13/83) for hCG and 86.21% (12/87) for GnRH, $p = 0.74$], but both differed from the control group [100% (0/103), $p < 0.001$]. There was no significant difference between groups in morula rates (4.71 ± 1.14 for the hCG group, 2.62 ± 1.54 for the GnRH group and 7.8 ± 2.33 for the control group). There was likewise no difference in blastocyst rates (1.71 ± 0.89 for the hCG group, 0.69 ± 1 for the GnRH group, and 3.2 ± 1.28 for the control group). We concluded that hormonal synchronization did not affect development of embryos after the morulae stage, but showed lower fertilization levels. This fact may have been due to the difference between the AI protocols used in the females of the control group and females who received hormonal treatment. No difference was observed in the groups using different ovulation inducers (hCG and GnRH).

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Prediction of non-pregnancy 20 days after ftai by corpus luteum evaluation using color flow Doppler

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Keywords: blood flow, corpus luteum, pregnancy.

The objective of this study was to determine the accuracy of color flow Doppler on prediction of non-pregnant animals 20 days after fixed-time artificial insemination (FTAI), using corpus luteum blood flow (CLBF) visual evaluation as the only parameter. Crossbred Holstein X Gyr, lactating dairy cows (n=129) and heifers (n=79) were synchronized for FTAI, in two replicates (R1, n=98; R2, n=110), using the following protocol: D -10 intravaginal implant (1.0 g progesterone, Sincrogest, Ourofino, Brasil) and 2 mg im estradiol benzoate treatment (Sincrodiol, Ourofino); D -2 implant removal and treatment with 0.5 mg im sodium cloprostenol (Sincrocio, Ourofino); D -1 treatment with 1 mg im estradiol benzoate; D0 – FTAI. Twenty days after FTAI (D20), animals were submitted to B-mode ultrasonography to locate the CL and color flow Doppler to evaluate luteal blood flow, using a portable ultrasound machine equipped with a 7.5 MHz rectal transducer (MyLab30 VetGold, Esaote, Italia). After Doppler mode activation, the operator visually evaluated the blood flow on the entire CL surface, from one side to the other. Based only on visual, subjective CLBF evaluation, animals were classified as 'pregnant' or 'not pregnant'. Thirty days after AI (D30), blinded from any information of the previous diagnosis (based on CLBF), the same operator performed a final pregnancy diagnosis (PD), using B-mode ultrasonography to visualize the fetal heart beat. The final pregnancy outcome (D30) was retrospectively compared to the diagnosis based on CLBF (D20) and the result of each animal was classified either as 'correct' or 'incorrect'. Numbers of true positive (TP), true negative (TN), false positive (FP), and false negative (FN) were inserted into a 2x2 contingency table. Sensitivity (SEN), specificity (SPEC), positive predictive value (PPV), negative predictive value (NPV), and accuracy (Acc) of the diagnosis based on CLBF were calculated using the following equations: SEN=TP/(TP+FN); SPEC=TN/(FP+TN); PPV=TP/(TP+FP); NPV=TN/(FN+TN); and Acc=(TP+TN)/total n. Binomial variables (pregnancy rate and proportions) were analyzed using the chi-square test. Overall pregnancy rate was 50.0% (104/208) and was not affected neither by replicate (49.0 vs. 50.9%, for R1 and R2; P>0.05) nor parity (51.9 vs. 46.8%, for cows and heifers; P=0.56). The performance parameters of the diagnosis based on CLBF were: SEN=97.1%; SPEC=51.9%; PPV=66.9%; NPV=94.7%; and Acc=74.5%. The proportion of false positive was 24.0% (50/208) and false negatives represented 1.4% (3/208). Considering that the primary objective of early PD is to identify non-pregnant animals to be re-synchronized, the conclusion is that visual evaluation of CLBF 20 days after FTAI is reliable for that purpose, due to its low proportion of false negative and high NPV.

Acknowledgments: FAPEMIG, CNPq, OUROFINO, EMBRAPA.

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Evaluation of corpora lutea vascularization by color Doppler during the period of maternal recognition of pregnancy: preliminary data

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Keywords: cattle, color Doppler, corpus luteum.

Progesterone production by the corpus luteum is closely related to blood flow. Consequently, evaluation of corpus luteum vascularization can provide important indexes of luteal function. The aim of the present study was to evaluate luteal vascularization between days 12 and 20 post-insemination in cattle. Holstein x Gyr crossbred cows and heifers (n=121) were inseminated using a FTAI protocol: D -10 insertion of a 1.0 g progesterone device (Sincrogest, Ourofino) and injection of 2 mg estradiol benzoate (BE, Sincrodiol, Ourofino); D -2 device removal and injection of 0.5 mg sodium cloprostenol (Sincrocio, Ourofino); D -1 injection of 1 mg BE; D0 – TFAI. The animals were evaluated each 48h from 12 to 20 days after FTAI, uzing a portable ultrasound device equiped with color Doppler (MyLab30, Esaote, Italia) and a linear rectal probe 7.5 MHz. The evaluation parameters were: total corpora lutea area (CLA), luteal tissue area (LTA, total area minus cavities area, if present), vascularization area (VA, established by Doppler signal) and VA/LTA ratio (VP). Pregnancy diagnosis was performed on day 30. The values of CLA, LTA, VA and VP between D12 and D20 were compared between animals later detected as pregnant or non-pregnant. Results are shown as mean±SEM. From the total of animals synchronized 17.3% (21/121) didn't ovulate and were excluded from the study. Pregnancy rate was 48.0% (48/100). The reference values of CLA, LTA, VA and VP on D12 were 3.98±0.17cm2; 3.61±0.12cm2; 0.50±0.04cm2 and 13.91±1.04%, respectively. There was no day effect on CLA, LTA, VA and VP in animals later diagnosed as pregnant (P>0.05). In non-pregnant ones, there was a reduction (P<0.01) in all luteal parameter values up to D20. Despite showing greater VCs (>50%), Doppler parameter (VA and VP) values were different between pregnant and non-pregnant animals earlier (D16: 0.66±0.04 vs. 0.42±0.04 cm2 for VA and 18.88±1.12 vs. 11.17±0.82% for VP, respectively, P<0.001) than LTA (difference after D18) or CLA (after D20). For the same day (D20), the magnitude of differences was also greater between Doppler and conventional measure values (231.20% [VA] and 171.46% [VP] vs. 25.79 [CLA] and 38.78% [LTA] of difference between non-pregnant and pregnant animals, respectively). These preliminary results suggest that the use of color Doppler can provide more precocious and more accurate diagnosis of luteal function changes during the period of maternal pregnancy recognition.

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Molecular evaluation of developmental competence of buffalo oocytes collected *in vivo* during winter and summer

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Keywords: buffalo, oocyte, season.

Buffaloes are polyestrous seasonal animals showing reduced fertility during summer at high latitudes. To investigate whether reduced fertility is related to oocyte competence, immature oocytes from buffaloes (nuliparous [N] and primiparous+multiparous [M]) were collected during winter (W) and summer (S) and subjected to molecular analyses. Sixteen female buffaloes (N=8 and M=8) at APTA-Registro/SP received in each season (July/2011 and January/2012) 3 mg norgestomet (Crestar, MSD, Cotia/SP), 2 mg estradiol benzoate (Gonadiol, MSD) and 530 µg sodium cloprostenol (Ciosin, MSD) at Day (D) 0. At D5, cutaneous (CT; oC) and rectal (RT; oC) temperatures and respiratory frequency (RF; breaths/min) of animals were measured before OPU. The PROC GLIMMIX of SAS (SAS Institute Inc., Cary, EUA) was used for analysis regarding a 2x2 factorial design and $P<0.05$. There was interaction effect for CT, number of follicles and mitochondrial DNA (mtDNA) amount; category effect for RT and number of oocytes (total e viable); and season effect for RT, RF and percentage of viable oocytes. No effect of ATP amount and oocyte diameter was found. CT was found to be higher in both N and M during S than W (NS=35.0±0.4; NW=23.8±0.5; MS=34.8±0.4; MW=25.4±0.5). CT was also higher in M than N during W. RT was higher during S than W (S=38.7±0.1; W=38.0±0.1) as well as it was higher in N than M (N=38.5±0.1; M=38.2±0.1). RF was higher during S than W (S=21.3±1.2; W=15.4±1.1). Follicle number (NS=22.6±2.6; NW=16.5±2.9; MS=13.4±1.9; MW=16.6±2.1) was increased during S than W in N as well as increased in N than M during S. Although the number of total (N=21.8±2.9; M=10.6±1.3) and viable (N=13.4±2.2; M=6.3±0.8) oocytes was greater in N than M, the percentage of viable oocytes (S=55.5±3.6; W=64.4±2.6) was lower during S than W. The amount of mtDNA (NS=263.673±51.367; NW=609.599±89.596; MS=648.935±99.728; MW=354.753±104.755) was decreased in oocytes from N during S than W and increased in oocytes from M during S than W. During S, the amount of mtDNA was lower in oocytes from N than those from M, but during W mtDNA amount was greater in oocytes from N than those from M. In summary, the majority of analyses performed do not suggest a negative effect of summer upon oocyte. More conclusive evidences of the season effect upon buffaloes oocytes are expected from on-going analyses of expression of genes related to mitochondria, apoptosis, glucose metabolism, oxidative stress, heat stress and oocyte competence.

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Flow-cytometry sex sorting does not affect the methylation pattern of the IGF2 gene in the bovine embryo

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Keywords: DNA, epigenetics, gene imprinting.

In mammals, correct DNA methylation reprogramming and maintenance of genomic imprinting after fertilization, are essential for embryo development and maintenance of pregnancy. Therefore, changes in DNA methylation, could affect subsequent embryo development and maintenance of pregnancy. Although in a recent study in our lab no effect of sexing by flow cytometry on the methylation pattern of the bovine sperm was detected, there are evidences that embryos produced with sexed sperm can have changes in methylation pattern. This possibility is supported by differences in gene expression and higher embryo loss between days 30 and 90 of pregnancy reported when sexed sperm was used. One important imprinted gene, related with embryo development, is the IGF2 gene. The objective was to investigate the effect of sexing by flow cytometry on the methylation pattern of the differentially methylated regions (DMR) located on exon 10 of the IGF2 gene in bovine embryos. One ejaculated from each Nellore bull (n=4) was collected and separated into three fractions: non-sexed (NS), sexed for X-sperm (SX), and sexed for Y-sperm (SY). Cryopreserved semen from each group was used in IVP. On day 9 (D9) of the development (D0=IVF), hatched blastocyst from each group were used to assess the methylation pattern of the IGF2 gene. Two pools of the 12 embryos produced with non sexed sperm and sexed sperm (pool of SX and SY embryos) were used. Each pool of embryos was used for genomic DNA isolation, which was used for analysis of the methylation by sodium bisulfite and sequencing. Methylation status was determined by the BiQ Analyzer software using the sequences in GeneBank NM_174087.3 as reference. For comparisons between groups, the Kruskal-Wallis test was performed ($P \leq 0.05$). No differences in DNA methylation were found between NS (29.8±3.7%) and pool of SX e SY embryos (18.3±3.4%). In conclusion, in vitro embryos produced with sperm sexed by flow cytometry did not affect the DNA methylation patterns of the DMR located on exon 10 of the IGF2 gene in bovine embryos.

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Sperm quality of post-thawed boar semen using different freezing curves

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Keywords: cryopreservation, semen, swine.

The boar sperm is susceptible to thermal shock during freezing and thawing being these events responsible for the low viability post-thawing. The objective of this study was to evaluate the effect of different freezing methods on sperm characteristics of boar semen. Twenty ejaculates from 20 animals were diluted in commercial extender and cooled at 17°C for 20 hours. The doses were centrifuged (1600g for 5 min. at 15 °C) to remove the supernatant. In methodology A (control - Westendorf et al., 1975, Deutsche Tierarztliche Wochenschrift, 82, 261-267), the pellet was resuspended in 1:3 semen: extender cooling (EC) (80% lactose solution 11% and 20% of egg yolk). Then, the samples were cooled to 5°C for 90 min. and then added two parts of freezing extender (EC 89.5%, 1.5% Ex Orvus Paste and 9% glycerol). The filling was done in 0.5 mL straws which were subjected to liquid nitrogen vapor (-90 °C) for 20 min. and kept in liquid nitrogen at -196 °C. In methodology B (biofreezer), the freezing extender was added just after the cooling extender, both in the same proportions described above. Doses were packaged into straws 0.5 ml and subjected to curve freezing ranging 15 °C to 5°C at 0.25°C/min., 5°C to -80°C at 10°C/min., and -80°C to -120°C at 5 °C/min. Thawing occurred in a water bath at 37°C for 20 seconds. The contents of each straw was diluted in 2.0 ml of ALMUS extender at 37°C and subjected to analysis of motility, vigor, membrane integrity (eosin nigrosin) and acrosome integrity (Pope staining). The data were submitted to paired t test, and statistical analyzes were performed in SAS statistical software (1996) at 5% probability. The motility (37.25 ± 2.98, 3.63 ± 37), the vigor (2.4 ± 0.14, 2.3 ± 0.105) and membrane integrity (61% ± 2.89, 60.25% ± 2.45) did not differ ($P > 0.05$) among the methodologies. The methodology A (45.9% ± 3.34) showed ($P<0.05$) higher acrosome integrity than the method B (40.45% ± 3.73), that can be explained by the fact that in the methodology A semen was only exposed to crioprotector at the temperature of 5 °C and not at 15 °C as in methodology B, in which can be occurred a cytotoxic effect. It is believed that the cooling curve of the methodology B was very slow, exposing spermatozoa for a long time in cryoprotectant prior to freezing. Thus, the method of freezing A is recommended, since it provided the best acrosome integrity.

A242 Supporting Biotechnologies: Cryopreservation and Cryobiology, Image Analysis and Diagnosis, Molecular Biology and "Omics"

Cryotolerance of *Bos taurus indicus* and *Bos taurus taurus in vitro* and *in vivo* produced embryos

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Keywords: bos indicus, bos Taurus, cryotolerance.

The objective was to study mechanisms involved in the post-cryopreservation survival of Bos taurus indicus (Nellore) and Bos taurus taurus (Simmental) in vitro (IVP) and in vivo (ET) produced embryos. In a 2x2 factorial experimental design, two subspecies (Bos taurus indicus vs Bos taurus) and two origins (PIV vs ET) were used. Nellore and Simmental cows (N=14) were submitted to OPU sessions and recovered oocytes (840 and 450, respectively) underwent IVM, IVF and IVC. Moreover, Nellore (N=7) and Simmental (N=8) cows were submitted to ovary superstimulation, FTAI, and uterine flushing. The IVP (N=349 and N=105) and ET (N=80 and N=74) embryos, zebuine and taurine respectively, were used in the vitrification (N=70-94), Sudan Black B stain (N=30), matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) (N=8-16), genomic microarray (N=4-8) and qPCR (N=8-16) techniques. Univariate (PROC LOGISTIC and PROC GLIMMIX, SAS 9.2) and multivariate (PCA - Piruete v.3.11 and MetaboAnalyst) models were used for the statistical analysis. Microarray data analysis was performed in FlexArray 1.6.1.1 software. Genes with a fold change of at least 1.5 and a probability of $P \leq 0.05$ were considered differentially expressed. Taurine embryos had higher ($P \leq 0.05$) post-vitrification survival (34.6 vs 20.2%). Nevertheless, taurine embryos had higher ($P \leq 0.05$) lipid content than zebuine (3.4±0.3 vs 2.4±0.3). When compared ET with PIV embryos, it was observed that the former had higher ($P \leq 0.05$) post-vitrification survival (38.5 vs 18.1%) and lesser ($P \leq 0.05$) lipid content (2.1±0.3 vs 4.0±0.3). Additionally, membrane lipids profile (phosphocholine- PC and sphingomielins- SM) were characteristic of each group, and potential positive [PC (34:2) and PC (36:5)] or negative [PC (32:0) and PC (34:1)] lipid biomarkers of embryo cryotolerance were established. Through microarray analysis, a total of 165, 520 and 54 genes were differentially ($P \leq 0.05$) expressed between subspecies, origins and subspecies*origins interaction, respectively. Genes involved in apoptosis (DAD1), lipid (AUH and ELOVL6) and mitochondrial (ATP5B) metabolism were upregulated in taurine embryos. GPX4 gene, involved in the oxidative stress, was upregulated in zebuine embryos. Genes involved in apoptosis (DAP), lipid metabolism (ACSL3 and ACSL6), maternal recognition of pregnancy - MRP (ITFN2), heat shock (HSPA5) and cell differentiation and placenta formation (KRT18) were upregulated in IVP embryos. In addition, there was a significant subspecies*origins interaction in the genes related to MRP (PAG2) and apoptosis (PRDX2). In conclusion, several metabolic pathways involved in cellular and embryonic signalization of different biological processes affect embryo post-criopreservation survival.

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A243 Supporting Biotechnologies: Cryopreservation and Cryobiology, Image Analysis and Diagnosis, Molecular Biology and "Omics"

Vitrification by cryotop vs. slow freezing: effect on re-expansion and hatching rates of IVP bovine embryos

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Keywords: blastocyst, criopreservation, efficiency.

Vitrification is the election method for cryopreservation of IVP embryos, and various techniques and protocols that use small volumes of vitrification solution have been developed and used. Among them, one approach is the cryotop, that although is widely used for oocytes, only few reports have evaluated its efficiency in bovine IVP embryos. The objective of this study was to evaluate the effect of vitrification by cryotop compared to the slow freezing regarding to reexpansion and hatching rates of IVP embryos. We used cumulus-oocyte-complexes (COCs) aspirated from slaughterhouse ovaries. After selection, the COCs were matured, fertilized and cultured *in vitro* to D7 of development (D0 = day of fertilization). Only D7 embryos at the expanded blastocyst stage (Bx) were used (total n = 144). The embryos were allocated into 3 groups: 1) control (CG), in which embryos remained on the bench, 2) vitrified (VG), embryos vitrified by cryotop method, and frozen (FG) embryos underwent slow freezing with ethylene glycol. During cryopreservation process of FG and VG, CG embryos remained on bench in medium (TCM-199 Hank's salt, Ivitrogen ®, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), on a heated stage at 37 ° C. After VG and FG warming, embryos from all the three groups were placed in SOF medium and returned to the incubator. Then, they were evaluated for reexpansion and hatching at 24 and 48 hours. Data were compared using Chi-square test ($P < 0.05$). At 24 hours post-thawed, no differences were observed ($P > 0.05$) in reexpansion (80.8% and 89.7%) and hatching rates (14.8% and 22.4%) for FG (n = 47) and VG (n = 49) embryos. When those were compared to the BG (n = 48), which showed reexpansion and hatching rates of 95.8% and 39.5%, respectively, only FG group presented a decreased in both rates ($P < 0.05$). At 48 hours, reexpansion rate showed the same behavior as observed at 24 hours. However, when hatching rates were evaluated, VG (71.4%) and BG (81.2%) groups were similar, showing higher ($P < 0.05$) percentage of hatched blastocysts than FG (42.5%). The results suggest that vitrification by cryotop is more effective to cryopreserve IVP embryos, when compared to the slow freeze technique. However, further studies are needed to evaluate the ability of cryopreserved embryos to establish a normal pregnancy.

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A244 Supporting Biotechnologies: Cryopreservation and Cryobiology, Image Analysis and Diagnosis, Molecular Biology and "Omics"

Variations in the protein profile of membrane-rich fraction of Saanen buck spermatozoa during dry and rainy seasons in northeastern Brazil

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Keywords: membranes, proteins, spermatozoa.

Membrane proteins play important roles during sperm maturation and capacitation, binding to the oviduct and fertilization. Knowledge about protein profile of sperm membranes will bring insights on how they affect sperm function. Therefore, the objective of this study was to investigate variations in the membrane proteins between dry (September, October and November) and rainy (March, April and May) seasons from Saanen bucks raised in Northeastern Brazil. The animals were housed indoors and fed the same diet throughout the experiment. Semen samples were collected biweekly from seven males and the seminal parameters evaluated. The sperm cells were separated from seminal plasma by centrifugation and stored at -20°C. Samples from each male were thawed and pooled by season. Sperm cells were washed with PBS and homogenized vigorously with crusher-type device. Then, broken cells were washed again with PBS, re-suspended in a buffer containing 1% Triton X-100 for 2 hours and maintained at 4°C. They were then subjected to sonication for 30 min and centrifuged at 5000 x g for one hour at 4°C. The supernatant was then precipitated with acetone and re-suspended in urea-thiourea buffer. Protein concentration was determined according to Bradford (1976, Anal Bioch 722, 248-254) and samples subjected to 2D electrophoresis. Values for semen parameters and protein maps between seasons were compared using paired Student's t test or Wilcoxon test, according to their distribution. Average semen variables for dry rainy and dry seasons, respectively, are shown in parenthesis. Sperm concentration (2.3x109 ± 0.13; 1.9x109 ± 0.09), percent of motile cells (54 ± 4.2; 30 ± 4.5) and percentage of morphologically normal spermatozoa (69 ± 3.8; 55 ± 3.6) were higher ($p<0.05$) in the rainy season. There were, on average, 238 spots per gel and the intensities of six among them differed ($p<0.05$) between seasons. These spots were cut, digested and identified by tandem mass spectrometry. ATP synthase protein subunit d (mitochondrial), radial spoke head protein homolog 9 and lysine-specific demethylase were more abundant in the rainy season. Their biological functions are related to ATP synthesis, flagellar movement and regulation of spermatogenesis, respectively. On the other hand, three isoforms of leucine aminopeptidase were more abundant in the dry season. This component is present in the acrosomal membrane and is involved in acrosome reaction. These changes in expression of proteins present in the membrane-rich fraction from goat sperm between different seasons suggesting that variations in environmental cues, such as ambient temperature and humidity negatively affect sperm quality acting by changing the expression patterns of proteins important for sperm function.

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RNA integrity of bovine reproductive tissues subjected to storage on ice and freeze-thaw cycles

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Keywords: cattle, reproductive tract, RNA degradation.

It is widely accepted that RNA molecules are unstable and highly prone to degradation. However, this concept has never been appropriately tested with the accurate and sensitive techniques currently available. The aim of this work was (1) to test the effect of time of storage of tissue and RNA extracts on ice and (2) to test the effect of freeze-thaw cycles of RNA extracts, on its integrity and detection of specific transcripts. Fragments from the endometrium (ENDO), corpus luteum (CL) and oviduct ampulla (AMP) were dissected from Nelore cows slaughtered on day 7 post estrus (n=3). Each fragment was sub-divided into 6 sub-fragments of 30 mg, which were stored in cryotubes on ice for 0 (one hour after slaughter), 1, 3, 6, 12 or 24 hours, and then stored at -80˚C. Total RNA was extracted using SV Total RNA Isolation System (Promega) and RNA integrity was assessed using the Bioanalyzer® apparatus. Time effect on RNA integrity number (RIN) values (1 = maximum degradation e 10 = absence of degradation) was analyzed by ANOVA. There was no difference among RIN values from ENDO (7.07±0.24; mean±standard error of the mean), CL (8.74±0.08) and AMP (6.86±0.3). Additionally, RNA extracts from each sample 1h were aliquoted and stored on ice for 0, 1, 12 or 24h or were submitted to 1, 2, 4 or 6 freeze-thaw cycles. There was no time effect on RIN values for samples of ENDO (7.7±0.09), CL (8.2±0.08) or AMP (5.9±0.7). Similarly, freeze-thaw cycles did not alter the integrity of RNA obtained from ENDO (7.6±0.07), CL (8.5±0.09) or AMP (5.8±0.6). Complementary DNA was synthesized (LifeTech) and the expression of progesterone receptor (PGR) and cyclophilin was analyzed by qPCR on RNA extracts stored on ice for 0 vs. 24h, and extracts that were subject to 1 vs. 6 freeze-thaw cycles. Mean differences between cycle thresholds (Ct) were determined by student's t test. Cycle threshold values for the PGR gene were altered neither by freeze-thaw cycles nor by time on ice, respectively, on ENDO (25.2±0.1 vs. 25.1± 0.1 and 26.9±0.4 vs. 27.4±1.7; Ct mean±standard error of the mean), CL (25.9± 3.4 vs. 29.8± 0.6 and 31.3± 1.1 vs. 30.4± 0.1) or AMP (29.3± 1.8 vs. 28.4± 1.0 and 31.3± 3.2 vs. 30.4± 2.0). Cyclophilin gene Cts were not altered on ENDO (21.5±0.1 vs. 27.0±1.5 and 28.9±2.8 vs. 26.6±0.9), CL (24.4±0.2 vs. 24.8±0.3 and 25.6±0.4 vs. 25.7±0.2) or AMP (28.4±1.1 vs. 27.9±0.6 and 29.2±1.8 vs. 28.9±0.8). In conclusion, it is suggested that neither generalized (measured by the Bioanalyzer) nor specific (measured by qPCR) RNA degradation occurs after tissue and RNA extracts storage on ice for up to 24h, nor after exposure of RNA extracts to up to 6 freeze-thaw cycles, on RNA obtained from bovine ENDO, CL and AMP.

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Supplementation with antioxidants during maturation increases resistance to oxidative stress in bovine embryos produced *in vitro* but does not improve cryotolerance

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Keywords: antioxidants, cryotolerance, in vitro maturation.

Since IVP bovine embryos are more susceptible to oxidative damage, *in vitro* supplementation with antioxidants has been shown effective to improve embryo quality. However, there remains to evaluate if the increase in quality is reflected in an increasing of cryotolerance of such embryos. This study was conducted to evaluate the effects of supplementation with antioxidants (cysteine – CIST; catalase – CAT; e β-mercaptoethanol – βME) in IVM medium on the development, quality and embryo cryotolerance, as well as the amounts of intracellular reactive oxygen species (ROS) produced during culture of bovine embryos. There were 5 replications, in independent replicates, and the blastocyst rate was calculated on the total of matured oocytes. COCs (n=766) were matured in B-199 (TCM-199 supplemented with bicarbonate, hormones and 10% FCS) supplemented with 0.6 mM CIST, 100 µM βME, 100 UI CAT or without antioxidants (Contr). After IVF, zygotes were IVC in SOF, at 38.5°C in 5% CO_2 in air, for 7 days. The cleavage rates and the embryonic development were evaluated, respectively, at 72 and 168 hpi. Part of obtained blastocysts (n=154) was vitrified (Ingámed®, Maringá-PR, Brasil). The remaining was stained (n=92) with 5 µM of the fluorescent probe 6-carboxy-2'7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes, Invitrogen, Oakville, Canadá) or stained (n=64) for TUNEL (In Situ Cell Death Detection Kit, Fluorescein, Roche Applied Science, IN, USA). Embryos stained with H_2DCFDA and TUNEL were evaluated immediately under an epifluorescence inverted microscope (excitation 495/510-550 nm and emission 520/590 nm, respectively) and the images of embryos stained with H_2DCFDA were analyzed by Q-Capture Pro image software for determining the fluorescent intensity. The embryos vitrified were thawed and cultured for 24 h to evaluate the re-expansion rates. The difference between groups was compared by ANOVA followed by Tukey's test and re-expansion rates by Chi-square test ($P<0.05$). The cleavage rates were 82.9%±3.2^a (Contr), 78.4%±2.1^a (CIST), 82.4%±5.2^a (βME) and 79.5%±3.5^a (CAT). The blastocyst rates were 48.7%±3.4^a (Contr), 53.9%±6.0^a (CIST), 51.4%±11.4^a (βME) and 59.7%±2.3^a (CAT). The re-expansion rates were 76.0%^{a} (Contr), 76.9%^{a} (CIST), 93.3%^{a} (βME) and 88.5%^{a} (CAT). The fluorescent intensity was 1.0±0.07^a (Contr), 0.8±0.05^b (CIST), 0.7±0.06^b (βME) and 0.6±0.06^b (CAT). The total number of cells were 85.7±3.5^a (Contr), 89.8±3.6^a (CIST), 89.8±3.2^a e 85.3±4.0^a (CAT) and the rate of apoptotic cells were 4.3±1.2^a (Contr), 2.2±0.4^b (CIST), 0.7±0.2^b (βME) e 1.8±0.3^b (CAT). In conclusion, antioxidant supplementation during IVM improved embryo quality by reducing intracellular levels of ROS and the rate of apoptosis, however, such improvement in embryo quality did not reflect in an increase in *in vitro* embryonic development and cryotolerance.

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Association of STR ILSTS002 and SNP FSHR in relation to different superovulatory response in embryo donors of Nelore and Angus breeds

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Keywords: bovine, molecular markers, superovulation.

Embryo transfer (ET) programs are important tools used to accelerate the selection process. Embryo transfer helps animals to obtain better performance of certain traits for different environmental conditions. It is important to have ET coupled with selection and breeding programs in order to obtain the best results. Molecular markers can aid reproductive techniques such as multiple ovulations, ET, oocyte maturation and in vitro fertilization. They allow the identification of specific genes introduced into the herd; however, it is necessary to study the association of these markers with the characteristics to be selected. This work aimed to investigate the association between STR ILSTS002, connected to the LHβ gene and a SNP linked to the FSH receptor (FSHR) in relation to different superovulatory response in embryos donor of Nelore (n= 60) and Angus (n= 59) breed. Data were obtained from donors with three programs for the collection of embryos, and for the marker ILSTS002 cows within each breed, were classified as high or low production of viable embryos in the case above or below 6 viable embryos for each collection. For the marker FSHR cows of each breed were divided into homozygotes (GG) or heterozygotes (CG) and compared the means of production of viable embryos. DNA was obtained by the salting-out method (Miller et al., 1988) and amplified using PCR with specific primers for the examined regions. A statistical model was performed to assess the association between the number of structures and the genotypes of each marker by univariate general linear model. In Nelore donors the presence of allele ILSTS002*135 was significantly high among animals classified as low producers (p= 0.04). In Angus no association was observed in relation to the different classes of donors. The genotypes analyzed for the SNP within FSHr demonstrated a frequency of 51.6% (CG) and 48.4% (GG) for Nellore animals. Interestingly, Angus animals demonstrated to be 100% (GG) homozygous for the SNP within FSHr. Nelore donors classified as heterozygous (CG) presented an average production of viable embryos significantly higher than those genotyped as homozygous 9.6 x 5.7 ($p < 0.01$). Based on our results we suggest the use of these molecular markers in the selection of donor cows of Nelore embryos in embryo transfer programs.

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Expression of genes associated with the oocyte competence of Nelore cows submitted or not to ovarian overstimulation

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Keywords: bovine, embryo transfer, gene expression.

Embryo transfer (ET) has contributed significantly to the genetic improvement of Brazilian herds. The superstimulatory protocol P-36 has been successfully used in ET programs. The objective of this work was to investigate the effect of P-36 protocol in the mRNA expression of genes positively correlated with the competence of cumulus-oocyte complex (COC): receptors of FSH (FSHR), EGF (EGFR) and pentraxin 3 (PTX-3) in cumulus cells; and also the mRNA of GDF-9, BMP15 and histone H2A (H2A) in oocytes. Purebred multiparous Nelore cows were randomly allocated into two groups: control and P-36. Animals of the control group (non-superovulated, n=15) received an intravaginal device containing progesterone (P4, 1.0 g, Primer®, Tecnopec) and 2.5 mg of estradiol benzoate (EB, IM, BER-BE®, Syntex) on a random day of the estrous cycle (D0). An analog of PGF2alpha (150 mg dcloprostenol, IM, Prolise®, RARS SRL) was administered on day 8 and the Primer® was removed. In the P-36 group (n=10) at the beginning of treatment (D0), cows received a primer® and 2.0 mg of EB. The administration of pFSH (total dose 160 mg Folltropin-V®, Bioniche Animal Health) was initiated on day 5 at decreasing doses: 40, 30, 20 and 10% of the total dose per day, divided twice daily for 4 days. On day 8 the PGF2alpha analog was administered and after 36 h the primer® was removed. Slaughter was performed 12 h (D9) after the primer® removal for ovary collection. Recovered oocytes were submitted to vortex and protease treatment (Protease®, Sigma-Aldrich) to remove the zona pellucida. Pools of 20 oocytes isolated and of their respective cumulus cells (n=6 pools; control group and n=4 pools, P-36 group) were subjected to total RNA extraction (RNeasy kit, Qiagen). The expression of target genes was performed by RT-PCR Real-time using oligo-dT in reverse transcription and bovine-specific primers. Expression of cyclophilin A (PPIA) was used as an endogenous control. The means of mRNA levels of target genes were compared by t test and significance was considered when $P < 0.05$. There was no difference in mRNA levels of FSHR, PTX-3 and EGFR in cumulus cells in both groups. However, in oocytes, the mRNA expression of GDF-9 and BMP15 was higher in the control group (0.68±0.21 and 0.68±0.24, respectively) when compared with the P-36 group (0.06±0.01 and 0.04±0.01, respectively, $P<0.01$). No effect of the superstimulatory treatment was observed on the mRNA levels of H2A in oocytes. Therefore, our results suggest that the oocytes of animals submitted to superstimulatory treatment seem to be less competent when compared to oocytes of cows not undergoing ovarian stimulation.

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Comparison of the expression of AKP2 in blastocyst and hatched blastocyst

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Keywords: Akp2, bovine blastocyst, pluripotency.

The investigation of the transcription factors involved in the regulation of pluripotency in preimplantation embryos of cattle provides important information regarding the early embryonic development and derivation of embryonic stem cells. The present experiment aimed to compare the level of expression of messenger RNA (mRNA) of pluripotency marker Akp2 (gene encoding alkaline fosfase) in blastocysts (D7) and hatched blastocysts (D10), because it is known that around the hatching of the blastocyst, inner cell mass (ICM) is transformed into a completely differentiated epithelium, the hypoblat and epiblast. Cumulus oocyte complexes were matured in TCM 199 for 24 h and fertilized with frozen – thawed sperm. Presumptive zygotes were cultured in SOFaaci for seven days (group 1) and for ten days (group 2). All embryos were washed three times in PBS, pooled and frozen at -80°C until RNA extraction. For the quantification of the mRNA expression levels of Akp2, total RNA was isolated from pools of 7 embryos per assay (n =3) and for each examined developmental stage using RNeasy Micro Kit (Qiagen). The reverse transcriptase Superscript III (Invitrogen) was used for the synthesis of complementary DNA and the polymerase chain reaction (PCR) was performed with the Gotaq qPCR Master Mix (Promega, MA, USA). The method used for quantification of expression was the relative standard curve method. The quantification was normalized to an endogenous control (the housekeeping gene YWHAZ), and standard curves for Akp2 and YWHAZ were derived from 10 – fold serial dilutions from bovine DNA and gave correlation coefficients greater than 0.99 and efficiencies greater than 94%. The data from three assays were analyzed by ANOVA and it was found that the level of transcription of mRNA of Akp2 is 5 times higher in blastocyst (D7) cells than in hatched blastocysts (D10) cells. As Akp2 is a pluripotency marker its expression is higher in earlier stage of embryonic development.

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Effect of reducing the concentration of ram semen on cryopreservation

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Keywords: concentration, cryopreservation, sheep.

In sheep frozen semen conventionally, a sperm concentration ranging from 80 to 150 million viable cells per dose is used, resulting in a small number of doses per ejaculate. Thus, males of high commercial value need to be submitted to a higher number of semen collections to produce a number of doses that can meet eventual demands. This study aimed to evaluate in vitro the effects of lowering the concentration of sperm per dose in cryopreservation of ovine semen, seeking to optimize the use of the ejaculates. Six ejaculates from two Dorper rams were collected by artificial vagina with one week interval between collections, being each ejaculate splited into four groups according to sperm concentration: G90 (control, $90x10^6$), G60 ($60x10^6$), G30 ($30x10^6$) and G15 ($15x10^6$). After processing, the cryopreserved samples were stored in cryogenic container for subsequent evaluation in vitro. The hypoosmotic test (HOST), slow thermoresistance test (TTR), assessment of sperm motility and vigor were made after thawing of the straws in water at 37 ° C for 30 seconds. Statiscal evaluation was made by analysis of variance and Tukey test for means comparion. The significance level was 5%. A descriptive analysis was performed using PROC MEANS of SAS 8. It was found that for fresh semen, the mean ± standard deviation of motility and vigor were 93.125 ± 2.47 and 4.83 ± 0.32, respectively. As for the frozen-thawed semen motility had a mean ± standard deviation of 11.32 ± 64.583, and the vigor 3.56 ± 0.34. Student's t test for was performed for paired samples between fresh and frozen semen for motility and vigor, in which a difference ($P < 0.05$) between the features was observed. To evaluate the influence of concentration of sperm per batch, Tukey test for ANOVA was made, using PROC GLM in SAS 8. In this case, from all combinations a difference ($P < 0.05$) was observed only for motility of frozen semen at a concentration of 15 million. Using the same test the influence of freezing on vigor at different concentrations was investigated, and no statistical differences were detected ($P < 0.05$). For analysis of motility and vigor in TTR using the t test, the mean ± standard deviation at time 0 was 47.92 ± 2.4 and 15.9 ± 0.49 and after 3 hours was 27.7 ± 13.5 and 1.64 ± 0.56, respectively, with no statistical difference between groups. Likewise data of HOST were evaluated. The results indicated that it is possible to reduce the concentration of insemination doses up to 15 million, without interfing in their cryopreservation. According to these results it is visible to the maintenance of sperm quality and viability at lower concentrations.

A251Supporting Biotechnologies: Cryopreservation and Cryobiology, Image Analysis and Diagnosis, Molecular Biology and "Omics"

Efficiency of ET of vitrified embryos in buffaloes

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Vitrification has proven more appropriate technique of cryopreservation of mammalian in vitro embryos. The present study aims to present novel outcomes of ET vitrified embryos in buffalo. Expanded blastocysts grade I, produced in vitro from OPU buffaloes donors were vitrified using the cryotop method on days 5, 6 and 7 of culture. In eight sessions, 70 vitrified embryos were transferred in the period of 01/02/11 to 02/04/12 to recipients previously synchronized at day 6 after ovulation (Embryo transfer fixed time protocol). All embryos were warmed in the lab and loaded into straws for ET and no temporary culture in CO2 incubators or selection of embryos was made. The pregnancy diagnosis by ultrasound was performed 30 days after ET. Confirmation and fetal sexing were made on day 60 of pregnancy. On day 30th and 60th, pregnancy rate was 37.1% (26/70) and 31.4% (22/70) respectively, being 12 females (54.5%) and 10 males (45.5%). Only 4 pregnancy losses (15.4%) were detected. These results are unprecedented in science and open new possibilities for the commercial use of OPU, IVP, ET and vitrified embryos in buffaloes.

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