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Bioinformatic approach to establish predictors of oocyte development competence in cumulus cells

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Keywords: biomarker, cumulus, oocyte.

One of the greatest difficulties in assisted reproduction techniques is the selection of high-quality oocytes for further fertilization and implantation. Consequently, the search for oocyte quality bioindicators is constant. Cumulus Oophorus is formed by a group of somatic cells surrounding the oocyte. These cells have been widely studied because of their intimate relationship with the oocyte during the entire folliculogenesis, oocyte maturation and ovulation processes. Cumulus cells gene expression and biochemistry are influenced by oocyte condition, as well as both follicular and ovarian environment. Still, these cells are discarded after in vitro fertilization techniques. This makes them an easy-to-access material, which can be obtained non-invasively and free of ethical biases. Our aim in this study was to identify, at cumulus cells level, oocyte competence related genes. To achieve this objective, we searched public repositories (Gene Expression Omnibus) for gene expression data from microarray obtained from cumulus cells experiments and which also provided clinical and pathological descriptions of the samples, including end-points of the germinative cell (cleavage, blastocyst formation, implantation). In these databases, we found patients samples comparing cumulus from follicles that originated good and bad quality oocytes (GSE55654, GSE54135, GSE37277, GSE22869, GSE18559, GSE9526), and also patients' samples with different pathological conditions, such as obese and polycystic ovary patients (GSE10946). To evaluate differences between experimental groups in these databases and obtain information about oocyte competence, we used three computational approaches: 1) differential expression analysis at R statistic environment using LIMMA package; 2) pathway enrichment utilizing GSEA (geneset enrichment analysis) method; and 3) Biological processes enrichment utilizing the online tool DAVID. Moreover, to reinforce and corroborate this approach and its results, we also used animal assisted reproduction studies (GSE65269, GSE36605, GSE31261). Our results, in addition to establish oocyte quality biomarkers, highlight the relevance of using cumulus cells to identify oocyte quality.

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Combination of hCG and deslorelin acetate on the induction of ovulation in mares: changes in follicular fluid protein profile

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Keywords: folicular fluid, ovulation-inducing, proteomic profile.

The composition of follicular fluid (FF) is essential for the proliferation and differentiation of granulosa cells in addition to processes related to rupture of the follicular wall, maturation and fertilization of the oocyte and luteinization. In the mare, there are few studies evaluating FF proteome (FAHIMINIYA, Prot Sci, 9:1, 2011; PETRUCCI, J Eq Vet Sci, 34:115, 2014). The ability to induce ovulation in a reliable way is important in equine reproductive management in different situations the main ovulation-inducing agents used are hCG and deslorelin. The aim of this study was to compare the protein profile of FF on induced ovulation of mares with hCG or with the combination of hCG and deslorelin acetate. Fourteen mares were used (3-12 years). Following the observation of follicles ≥ 35 mm and with endometrial edema, the mare was submitted to the induction protocols: Group H 1000 UI, IV, of hCG or Group HG 1000 UI hCG, IV, + 1,5 mg of Deslorelin acetate, IM. In the subsequent cycle mares were submitted to a protocol different from the previous cycle. Samples were collected by transvaginal aspiration 32 h after induction and submitted to the quantification of proteins by the Bradford method. Two-dimensional electrophoresis was performed in 12.5% polyacrylamide gel and stained with Coomassie G250, scanned and analyzed using PDQuest v.8.0.1. Spots with significant differences in relative abundance between group H and HG were cut out submitted to trypsin digestion and mass spectrometry. In this study the total protein concentration in the mare FF from Group HG were higher (73.07 ± 6.42 mg/ml) than those induced with hCG alone (63.97 ± 6.97 mg/ml). Comparative analysis showed a significant difference in the abundance of five spots between groups. Two Alpha-1-antiproteinase 2 (A1AT2), the Serotransferrin (TF) and Antithrombin III (ATIII) had lower relative expression in group HG and the Haptoglobin (HP) showed greater abundance in the same group. The lowest expression of A1AT2 at the final moment of follicular maturation prior to ovulation is likely related to the need for lower inhibitory action on the proteolytic activity allowing fine adjustment that controls the ECM degradation, inflammation and the coagulation cascade (BIANCH, J Prot, 90:61, 2013). ATIII is also serine-type endopeptidase inhibitor activity. The last protein less expressed in the group HG was TF. Increase in cellular iron levels stimulates the expression of some MMPs that degrade the ECM. There are reports of increased transferrin in granulosa cells and oocyte follicles in more advanced stages of maturation, which could explain the reduction in FF transferrin. Haptoglobulin showed increased abundance in the group HG and exerts anti-inflammatory action due to inhibition of oxidative damage. These proteins are probably related to the final events of oocyte/follicle maturation that trigger ovulation and subsequent luteinization.

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Evaluation of *in vitro* embryo production rate and re-expansion post thawing with two different culture media

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Bio Biotecnologia Animal.

Keywords: cryobiology, cryopreservation, cryotolerance, vitrification.

The in vitro embryo production is widespread in Brazilian dairy and beef cattle. However, the cryopreservation of IVF embryos remains a major step to be done for the IVF employment in an exponential scale. The aim of this work was to evaluate the effect of supplementing in vitro culture with BSA during the first 72 hours of bovine in vitro culture (IVC), on their embryonic development and cryotolerance. Slaughterhouse-derived oocytes were matured and fertilized in vitro according to standard procedures. Twenty-four hours after in vitro fertilization, zygotes were cultured in two different groups: Group Control, IVC media supplemented with 10% BFS from D.0 to D.7; Group BSA-BFS, from D.0 to D.3, IVC media + 0.6% BSA and, D.3 to D.7, IVC media + 10%SFB. Embryo development rates were evaluated on D.7, when the expanded blastocysts (Stage 7) were vitrified. Embryo cryotolerance was evaluated 48 hours after thawing, by the percentage of embryos resuming development to reach a more advanced stage, and hatching rate. BSA during the first 3 days decreased the blastocysts yields comparing with Control Group [23%a (146/637) vs 34%b (236/700), respectively; $p \leq 0.01$]. However, BSA improved embryo cryotolerance compared to the Control Group, due the higher development and hatching rates [88%a (63/72) vs 77%b (66/86), respectively; $p \leq 0.05$] after 48 hours post-warming culture. In conclusion, although the use of BSA during the first 72 hours of IVC decreases the embryo production, it improves the embryo cryotolerance.

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Evaluation of DNA amplification kits for genomic analysis of cell biopsies from *in vitro* produced bovine embryos

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Keywords: biopsy, embryo, genomic.

Cattle producers are continually looking for ways to increase the number of genetically superior animals, while at the same time reducing production costs. A genetic screen of in vitro produced embryos for traits of economic importance, prior to transfer, would greatly benefit genetic selection and reduce costs. This technique involves removal of a few cells from the growing embryo at the morula or blastocyst stage. The limitations of whole genome analysis (WGA) of embryo biopsies is to balance the number of cells that can be removed without compromising the embryo and obtaining sufficient amounts of DNA. With the development of molecular biological techniques such as DNA amplification, these limitations are no longer an issue. The objectives of this project were to evaluate: 1) the effects of biopsy and vitrification in a 2x2 factorial design on embryo re-expansion, and 2) the call rates of DNA from embryo biopsies that were amplified with commercially available DNA amplification kits. In experiment 1, at day 7 of in vitro production, bovine embryos either not manipulated (C) or subjected to embryo biopsy (B), in which around 10 cells from the trophoblast layer were removed. Both groups were either placed back into culture for 18 hrs (Fresh: C/F and Biopsied/Fresh: B/F) or cryopreserved by vitrification (C/V and B/V). Vitrified embryos were then thawed and placed into culture for 24 hrs. Re-expansion was evaluated in all groups. In experiment 2, DNA from biopsied embryos was amplified by GenomePlex Single Cell WGA (Sigma), REPLI-g Single Cell (Qiagen) or Illustra Single Cell GenomiPhi (Ge Healthcare) kits. Whole genome analysis was performed on the Geneseek Genomic Profiler LD (GGP-LD) for Indicus-35,000 SNP Platform (Neogen). One-way ANOVA test was utilized to determine significance ($P<0.05$) for re-expansion and call rates and differences in means was determined by Tukey`s. Re-expansion of B/F embryos (93.3+4.6%) was no different than C/F group (100%). Re-expansion of B/V embryos (75.0+6.7%) was lower than C/F, with C/V embryos having the lowest re-expansion rates (52.2+ 15.5%). There was a significant difference in call rates for DNA amplified using the REPLI-g (86.6+5.2%) and GenomiPhi (59.9+7.8%; $P<0.05$) kits. The GenomePlex kit (82.7+2.1%) was not different than the other kits. These results suggest that whole genome analysis of embryo biopsies is possible with DNA amplification without compromising embryo survival.

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In vitro evaluation of hyperactivation induction with procaine and calcium ionophore in cryopreserved equine sperm

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Keywords: freezing, frozen semen, sperm capacitation.

The aim of the present work was to test 2 in vitro hyperactivation protocols of cryopreserved equine sperm. After semen thawing, the sperm were selected by swim-up and distributed in 3 aliquots according to the treatments T1) Control: capacitating Whitten's Medium (WMc), T2) Procaine: T1 + 5mM Procaine chloride (Sigma-Aldrich, St Louis, MO, USA) e T3) Calcium Ionophore: T1 + 5µM Calcium Ionophore A23187 (Sigma-Aldrich, St Louis, MO, USA). After 10 min at 37°C, the samples were diluted to10x106 sperm/mL and the sperm motility was verified by a computerized assisted sperm analysis (CASA). To the bovine zona-binding assay (ZBA), bovine oocytes derived from abattoir ovaries were stored in a hyperosmotic solution (Yanagimachi et al., Fertil Steril, v.31, n.5, p.562-574, 1979), and the equine sperm, in the 3 before mentioned treatments, were stained with 35µg/mL Hoescht 33342 dye (Sigma-Aldrich, St Louis, MO, USA) and resuspended with WMc to 2x106 sperm/mL. The oocytes were incubated at 38.5°C with 5% CO_2 for 1 hour and each 5 oocytes were poured into each treatment droplet under mineral oil (Coutinho da Silva et al., Reproduction, v. 143, p. 577–585, 2012). After 2 hours of co-culture, the number of sperm attached to the ZP was determined with epi-fluorescent microscopy. The experimental design was performed completely randomized. Means of zona pellucida (ZP) attached sperm were compared by ANOVA and Tukey Test, the VCL, VSL, VAP, LIN, STR sperm characteristics by ANOVA, Tukey test and Scott-Knott, and ALH and BCF by Friedman's Test. The complete data were analyzed using the SAS program. A probability of $P<0.05$ was considered significant. Lower number of ZP attached sperm was observed by the calcium ionophore induced hyperactivation protocol (1.9±2.1) compared to the procaine (5.9±3.7) and control (5.7±3.6, $P<0.05$). The procaine hyperactivated sperm showed the lowest VSL, VAP e LIN values, while ALH e BCF did not differ from the control and calcium ionophore hyperactivated sperm ($p>0.05$). It can be concluded that equine cryopreserved sperm were better hyperactivated with procaine than with calcium ionophore, and therefore, it is a more suitable sperm hyperactivation inductor to study equine IVF protocols with cryopreserved semen.

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Lipid characterization by Maldi-MS of *in vitro* produced bovine embryos with different developmental kinetics

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Keywords: bovine embryo, lipids, mass spectrometry.

Embryo studies have shown that morphological differences related to the moment of the first embryonic cell divisions and its kinetic (fast and slow), appear to be relevant to the embryo viability (Meseguer, M, Hum. Reprod, 26, 2658-71, 2011; Lundin, K, Hum. Reprod, 16, 2652-2657, 2001). Embryonic viability can also be severely compromised by lipid accumulation in IVP embryos and can even harm commercial applications such as cryopreservation (Abe, H, Mol. Reprod.Dev, 61, 57-66, 2002). Therefore, the aim of this study was to evaluate the relation between developmental kinetic and the lipid profile on bovine embryos. For this goal, IVP embryos were produced and, 18 hours post insemination (hpi), the presumptive zygotes were individually cultured for sequential medium (established in previous studies). First, they were cultured for 22 hours in KSOM (Millipore®) [supplemented with 10 % of FCS, 0.25µL/mL of gentamicin and 4.5µL/mL of nonessential amino acids] and classified as fast (F - 4 or more cells) or slow embryos (S - 2-3 cells). The medium was then replaced by SOF [containing 5% of FCS, 10µL/mL of nonessential amino acids and 20µL/mL essential amino acids] and the embryos were IVC until blastocyst stage (168hpi). The study also included a group of *in vivo* (VBL) embryos at the blastocyst stage. The lipid characterization, by MALDI-MS, was performed at 40hpi (FCL vs SCL), 96hpi (FMO vs SMO) and 168hpi (FBL vs SBL vs VBL). The lipid characterization data obtained was submitted to multivariate statistical analysis, by Partial Least Squares- Discriminant Analysis (PLS-DA), using the MetaboAnalyst 3.0 website. The ions highlighted were subjected to ANOVA with subsequent Tukey test (GraphPad Prism Software Inc 5), to show their relevance in the study groups. Fast and slow groups presented different relative abundances of membrane sphingomyelins (SM) and phosphatidylcholines (PC) by MALDI-MS at 40 hpi (of *m/z*: 725.5, 732.6, 758.6, 788.6, 808.6, with $p<0.05$) and 96hpi (of *m/z* 706.5, 725.5, 782.6, with $p<0.05$). Regarding the blastocyst stage, the slow group presented a higher relative abundance of the SM and PC species, which are similar to the VBL group (of *m/z* 703.5, 760.6, 784.6 and 808.6, with $p>0.05$). The slow group still showed some similarities with the fast group, which may be due to the exposure to the same *in vitro* environment (SM of *m/z* 703.5 and PC of m/z 734.6, for 96 hpi. PC of *m/z*: 786.6 and 808.6, for 168 hpi, $p>0.05$). In addition to their structural function, these lipid classes have an important role in cell signaling such as stress, pregnancy and cryopreservation. The ion data obtained for the PCs and SMs classes, allowed us to infer that there are more similarity between the SBL and VBL.

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Characteristics of H3K4me3 in bovine embryos produced *in vivo* with sexed semen and submitted to cryopreservation

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Keywords: epigenetics, histone methylation, sexing of semen.

Cryopreservation of embryos produced *in vivo* is considered efficient and the knowledge of its basic biology can be used as a parameter for better understanding of the mechanisms involved in the low efficiency of cryopreservation in embryos produced in vitro. The objective of this study was to characterize the modifications of tri-methylated lysine 4 on histone 3 (H3K4me3) in bovine embryos produced in vivo with sex-sorted semen and subjected to cryopreservation. Girolando cows (a common Brazilian dairy breed, crossbreed Gir x Holsteins; n = 5) were superovulated and inseminated with sex-sorted or conventional semen. After the embryo collection (n = 4), aliquots of the collected embryos were stored in Paraformaldehyde immediately after collection, or stored in the same medium after cryopreservation / thawing. All embryos (fresh / conventional semen, fresh / sexed semen; cryopreserved / conventional semen and cryopreserved / sexed semen) were evaluated by immunofluorescence in confocal microscopy to identify the H3K4me3. From 190 embryos collected, 100 were produced with conventional semen and 90 with sexed semen. The use of conventional semen provided 72% of viable embryos, the majority (52.77%) of them was in more advanced stages of development (expanded blastocysts; 52.77%). In contrast, embryos produced with sexed semen showed lower viability rate (36.7%) and earlier stages of development at time of collection (morula and blastocyst, 42.42%; $p < 0.05$). Embryos in morula stage, regardless of cryopreservation or the use of sexed semen, showed the most prevalent signal of the H3K4me3 compared to blastocysts (209.26 ± 10.95 vs. 160.35 ± 10.52 intensity channels; $p < 0.05$). These are the first results that describe the H3K4me3 profile of bovine embryos produced in vivo. As well fresh embryos as cryopreserved, and produced with conventional or sexed semen showed the same pattern of trimethylation of H3K4, but in morula the abundance was more pronounced.

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Lysine 4 of histone 3 trimethylation characteristics in bovine oocytes matured *in vitro* and cryopreserved - preliminary results

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Keywords: cryotop, epigenetic, H3K4.

It has been reported that immature oocytes are more sensitive for vitrification then mature. Factors such as maturity may change the pattern of DNA methylation and histone, compromising embryonic development. Accordingly, it is believed that the cryopreservation of oocytes can be influenced by epigenetic changes. Therefore, the aim of the study was to characterize the pattern of trimethylated H3K4 (H3K4me3) in mature and immature oocytes before and after vitrification. Oocytes were obtained by follicular puncture of bovine ovaries from slaughterhouse, later screened and classified according to their morphology and then divided into four groups: Group 1 = immature fresh (n = 15); Group 2 = immature cryopreserved (n = 15); Group 3 = mature fresh (n - 15); Group 4 = mature cryopreserved.The groups 3 and 4 were matured for 24 hours in incubator (5% CO_2, 38.5°C). Groups 2 and 4 were vitrified by cryotop method. All groups were kept in 3% PAF at 4°C to evaluate the H3K4me3, the oocytes were exposed to polyclonal primary antibody against H3K4me3 (CellSignaling®; 1:1000), and secondary antibody (anti-Rabbit Alexa-647; Invitrogen A21245, 1:200), and then subjected to staining of the nucleus (SYBR Green, Molecular Probes, Goettingen, Germany; 1:500). Finally, the oocytes were evaluated under confocal microscopy. The signal was analyzed by using the ImageJ program. Data were analyzed by ANOVA (factorial), and when necessary carried out Tukey test at 5% probability (R Program). The mature oocytes showed higher signal intensity (193.43 pixels) that immature (149.00 pixels) ($p<0.05$) different than expected, since the transcript in that period is known to be reduced and also supported by the fact that DNA methylation is high in mature oocytes. Fresh oocytes (the ones that have not undergone vitrification), on the other hand, showed higher signal intensity (178.00 pixels) than vitrified oocytes (164.43 pixels) ($p<0.05$). This fact was expected, as cryopreservation can cause injuries that culminate with the lowest transcriptional activity, which could interfere with the quality of oocytes and their ability to become a viable embryo. Hypothesis supported by the lower embryonic development rate of cryopreserved / thawed oocytes. The interaction of the variables was not observed ($p>0.05$). We can conclude, therefore, that maturation and cryopreservation of bovine oocytes affect the trimethylation of H3K4.

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Comparison of pregnancy rates after fresh, vitrified or cryopreserved *in vitro* produced embryos for direct transfer

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Keywords: cryopreservation, direct transfer, IVF embryos.

Although over 30% conception rates have recently been achieved from embryo transfer (ET) of vitrified in vitro produced (IVP) embryos, the complex process of recovery of these embryos after vitrification remains an obstacle to commercial use of this technique, with little applicability under field conditions. The aim of this study was to compare pregnancy rates obtained after ET of fresh IVP bovine embryos, vitrified or frozen for direct transfer in the commercial routine of a big dairy farm. Oocytes (n=3171) recovered by OPU from 120 Girolando females were selected and submitted to IVM for 24 hours at 38.5°C with 5% CO_2 in air and saturated humidity. The IVF was performed with sexed semen thawed from 5 Holstein bulls. After IVF, the presumptive zygotes were denuded and cultured for seven days under the same conditions for temperature and humidity as IVM and IVF, but with 5% CO_2 and 5% O_2. Grade I embryos in stages BL or BX were transferred to fresh, frozen or vitrified for direct transfer (DT). Embryos were transferred into previously synchronized recipients. The pregnancy rates was analyzed by Binomial Logistic Regression and the probability level of $p<0.05$ was considered significant. Conception rates obtained were 51.35% (133/259) for embryos transferred fresh, 34.62% (84/234) for vitrified and 42.11% (96/228) for direct transfer embryos. The rates obtained from IVP embryos vitrified and direct transfer were not different between these two groups, but they were lower than the pregnancy rates from the fresh group. These results indicate that cryopreservation of IVP embryos yields results similar to those obtained after transfer of IVP fresh embryos. These results highlight the positive aspects of the possibility of cryopreservation of IVP embryos with the convenience of direct transfer.

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Comparison between Bioxcel® and Tolera-D® semen extenders on semen industrialization rates

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Keywords: cryopreservation, flow citometry, semen.

Male fertility is always put to the test when undergoing a bull was submitted for semen collection and cryopreservation processes. There are several different factors that interfere on this; the quality of semen extender is one of them. The goal of this paper was compare de approval semen industrialization taxes (refused versus approval semen bath for commercialization) process with two different commercial semen extenders (Bioxcell® and Tolera-D®). Six semen donors were used (03 Nellore, 01 Angus and 02 Senepol bulls), collected by electroejaculation or artificial vagina. The ejaculates were evaluated by volume, mass motility, motility, vigor and morphology. After initial approval the ejaculates were split in 2 equal parts, one diluted with Tolera-D® (Inprenha, Jaboticabal, Brasil) and another with Bioxcell® (IMV, L'Aigle, França). After dilution, the semen bath was submitted by cooling, equilibration and freezing curve routinely used by the company. Semen was frozen in 0.25mL semen straw. Four straws randomly choose for each semen bath were chose for evaluation. After thaw (35oC in water), the semen batch were analyzed in contrast microscopy (100x and 1000x) by motility/vigor at thaw (MOT/V0h), motility/vigor after 3hs at 37ºC incubation (MOT/V3h), motility/vigor after 30' at 45ºC (MOT/V30) and % of viable sperm cells by flow cytometer-MUSE® (%VIACOUNT). The VIACOUNT test was design following the manufactory recommendations, with dilution factor (121X) and 10.000 events considered. The acceptable pattern to approval frozen semen batch was MOT/V0h >40/4; MOT/V3h >20/2; MOT/V30 >20/2; % VIACOUNT >40%; Concentration >20x106sperm/dose, total morphological defects <30% or major defects <20%. The approval rates of semen freezed using Tolera-D was 83% (100% in Nellore and Angus bulls and 50% of approval in Senepol breeder). Even the Bioxcell approval semen industrialization rates were 67% (83% in Nellore, 0% Angus and 50% in Senepol bulls). The results showed no statistical difference between extender's and approval semen industrialization taxes (Bioxcell® ou Tolera- D®), but we just note some particularities between some individuals and his semen behavior using different extenders. Both extenders have similarities between chemical composition and crioprotectors (glycerol and soybean lectins) and they demonstrated good and similar approval semen industrialization tax.

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Cryopreservation of bovine fibroblasts in straws after submission to negative pressure

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UDESC.

Keywords: controlled stress, cryopreservation, somatic cells.

The somatic cell cryopreservation plays an important role not only to aid in germoplasm preservation, but also in keeping genotypes used for experiments on induction of pluripotency, cell lineage formation and cloning by somatic cell nuclear transfer. The study of the effects of controlled stress in gametes and somatic cells has demonstrated that under certain experimental conditions, it can provide a higher survival rate, as well as it might play a role in parameters such as the growth pattern in culture. To assess that, bovine fibroblasts at first passage, obtained by standard procedures of cartilage explantation were randomly allocated into 4 experimental groups, as described: Fibroblasts were submitted (in culture) to 500 mBar of negative pressure during 4 minutes immediately (PN0h) or 3 hours (PN3h) before freezing (in standard procedures using 10% DMSO in D-MEM). After tripsinizing, cells were loaded into 0.25 mL straws, at a concentration of 1 x 106 cells/mL and 200 µL of total volume. The straws were sealed and stabilized in conventional fridge (5-7 ˚C) for 30 minutes, then exposed to nitrogen vapor (4 cm above the surface) for another 5 minutes and finally plunged. Thawing was performed in water-bath at 36°C for 20 seconds. Non-treated fibroblasts were used as fresh (FC) and cryopreserved (CC) controls. The parameters assessed were post-thaw viability, replication index (based in the cellular population doubling time - PDT), with intervals of 24 h and 8 days of length. At every session, one well of each group was tripsinized and the number of viable cells was estimated with the aid of haemocytometer after staining with trypan blue. The PDT was determined using the algorithm available online (http://www.doubling-time.com): TD = t x lg2/(lgNt – lgN0). Data were analyzed through ANOVA and T-test, with 5% of significance. The average cell survival rate of the groups CC (89.8%) and PN0h (88.1%) were higher than for the group PN3h (68.7%). The PDT length was similar among the groups FC (27.5±0.35 h); CC (30.1±2.3 h) and PN0h (32.4±1.6 h), with tendency (P=0.06) of an increase of PDT length for the group PN3h (34.8±2.3 h), in comparison to FC. Plastic straws were suitable for freezing bovine fibroblasts. No negative effects were observed after submitting the cells to negative pressure immediately before freezing. The culture for 3 h after the negative pressure reduces the growth rate of the cells, after freezing/thawing. New studies might be conducted, aiming to evaluate whether the treatment with negative pressure affects either the senescence pattern of the cells, or the viability of cloned embryos produced using negative pressure-treated somatic cells.

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Lipid dynamics in early bovine embryo development

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Keywords: early embryonic development, lipidomics, pre-implantation.

Mammalian pre-implantation embryonic development is a complex, conserved and well-orchestrated process involving dynamic molecular and structural changes. Understanding membrane lipid profile fluctuation during this crucial period is fundamental to address cellular and molecular mechanisms governing embryogenesis. Full understanding of stage-specific lipid signatures in early bovine embryo development is however still lacking. The primary aim of the present work was therefore to monitor the dynamic changes in stage-specific lipid profiles during early bovine embryonic development. For that purpose, MALDI-MS was used, and the observed changes were associated to the amount of cytoplasmic lipid droplets. Immature oocytes (-24 h post-insemination [hpi]) were recovered from slaughterhouse-derived ovaries, and 2-cell (32-40 hpi), 8 to 16-cell (72 hpi), morula (120 hpi) and blastocysts (168 hpi) were in vitro produced using two different culture media (SOFaaci supplemented with 2.5% of serum and serum-free SOF-BE1). For statistical analysis, uni- and multivariate models were used. Lipid content, monitored by lipid staining (n= 5-9 per stage), increased ($P<0.05$) at morula followed by a sharp drop ($P<0.05$) at blastocyst stage (58.4 ± 10.5^{ac}, 62.5 ± 9.4^{ac}, 85.9 ± 8.2^{a}, 148.3 ± 7.4^{b}, 37.4 ± 9.9^{c}; respectively for immature oocyte, 2-cells, 8 to 16-cells, morula and blastocyst). Cytoplasmic lipid droplets have increased ($P<0.05$) by SOFaaci at morula (162.6 ± 11.3 vs 137.1 ± 9.2) and blastocyst (49.9 ± 9.9 vs 20.7 ± 9.9) stages compared to SOF-BE1. Differences in the phospholipids profiles as monitored by MALDI-MS (n=5-9 per stage) were addressed by multivariate analysis. Characteristic dynamic changes were observed during early embryo development for the phospholipid profiles (phosphatidylcholines, sphingomyelins, and phosphatidylethanolamines), which were significantly related to unsaturation level, acyl chain length and class composition. This study provides a comprehensive analysis of stage-specific lipidome signatures in early bovine embryo development. The present data should be useful to assess the role of specific lipid species in important events of embryogenesis as well as to improve outcomes in both bovine and human assisted reproductive techniques.

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Effect of seminal plasma addition of whole and vasectomized rams to semen after collection

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Keywords: addition, ram, seminal plasma.

The seminal plasma could have a protective effect when added to thawed semen. Little is known about its effect in addition to semen immediately after collection and if seminal plasma of high freezability semen collected from whole animals and vasectomized rams, can increase the cryopreserved semen of low semen freezability rams. The objective of this study was to evaluate the addition of seminal plasma collected from whole ram (INT) and vasectomized ram (VAS) of high freezability semen in semen of low freezability before the cryopreservation process. Four rams of low freezability semen were used as semen donors and two rams with high freezability semen as seminal plasma donors. Three treatments were used: VAS – Addition of 30% of seminal plasma from vasectomized ram in the middle of pre-dilution; INT – Addition of 30% of seminal plasma from whole ram in the middle of pre-dilution; and control (CONT) - without addition of seminal plasma. We evaluated the subjective motility, total motility and progressive motility by CASA and membrane integrity after thawing (TIME 0) and after the thermal resistance test (TRT) for 4 hours (TIME 4). For statistical analysis by calculating average and standard deviation of the parameters analyzed, followed by analysis of variance using the GLIMMIX procedures, and the comparison of averages made by Tukey test corrected for multiple comparisons. It used the SAS Enterprise 5.1 program. After thawed (TIME 0) the parameters subjective motility, total motility, progressive motility and membrane integrity in the VAS treatment (59.72 ± 3.4, 60.08 ± 2.6, 40.94 ± 3.4, 39.52 ± 2.7) and INT treatment (49.84 ± 2.9, 49.74 ± 3.6, 31.79 ± 3.4, 30.95 ± 2.6) was higher ($P<0.05$) than the CONT treatment (37.90 ± 2.5, 36.58 ± 3.5, 22.12 ± 1.9, 2.7 ± 21:18). With regard to the parameters after the TRT (TIME 4) subjective motility in VAS treatment (50.78 ± 3.6) and INT treatment (40.02 ± 4.0) was higher ($P <0.05$) than the CONT treatment (27.88 ± 2.6). As for the parameters of total motility, progressive motility and membrane integrity VAS treatment (51.92 ± 3.5, 35.46 ± 3.4, 38.78 ± 1.4) was higher ($P <0.05$) than INT treatment (38.86 ± 2.9, 24.36 ± 3.5, 24.11 ± 2.0) and CONT treatment (28.91 ± 2.2, 12.19 ± 3.0, 02.11 ± 1.6) and the INT treatment was higher ($P <0.05$) than CONT treatment. The addition of seminal plasma before the cryopreservation process, can improve the resistance of damage caused in cryopreserved ram semen, with seminal plasma collected from vasectomized animals having best results.

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Effect of adding conjugated linoleic acid to *in vitro* culture medium in the viability after vitrified bovine embryo transfer

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Keywords: conjugated linoleic acid, embryo transfer, vitrification.

In vitro produced embryos exhibit low cryotolerance due to their high concentration of cytoplasmic lipids (Abe et al., J. Reprod. Dev., v.49, p.193, 2003). The conjugated linoleic acid trans-10, cis-12 (CLA) decrease lipogenesis in cells, improving embryo quality and possibly decreasing its sensitivity to cryopreservation (Mitchell and McLeod, Biochem. Cell Biol., v.86, p.293, 2008). It was evaluated the effect of adding CLA to in vitro culture medium in the viability after vitrified bovine embryo transfer. Three culture media were used: Control (n=340 oocytes): SOF medium added to BSA and FBS, without the addition of CLA; FBS+CLA (n=359 oocytes): SOF medium added to BSA, FBS and CLA; CLA (n=339 oocytes): SOF medium plus BSA added to CLA, without adding FBS. The blastocysts were submitted to the Open Pulled Straw vitrification method for subsequent heating. The recipient estrus was synchronized using protocol with intravaginal progesterone device (1.9g), associated with estradiol benzoate (2.0 mg) and estradiol cypionate (1mg), eCG (250UI) and analog PF2α (0.05 mg of sodium cloprostenol). There were transferred one or two embryos to the uterine horn ipsilateral to the corpus luteum (CL): T1 [recipients that received one blastocyst (n=17 embryos, Control=5, FBS+CLA=6 and CLA=6)]; T2 [recipients that received two blastocysts (n =54 embryos, Control=18, FBS+CLA=14 and CLA=22)]. In D7 after induced estrus the recipients were evaluated for CL confirmation. 58% of the animals responded with exacerbated form to estrus induction protocol, with multiple ovulations (2 to 7 CLs) and multifollicular growth (follicles up to 1.9cm and follicles larger than 2.0cm). The embryo transfer was performed only on animals with CL and follicles smaller than 1.9cm. To assess viability, vitrified embryos were heated (Control=27; FBS+CLA=30; CLA=17) and cultured in vitro to verify the reexpansion and hatching ability after 24, 48 and 72 hours of cultivation. There was no difference in blastocyst production relative to the total number of oocytes fertilization (37.1% for Control; 35.4% for FBS + CLA, CLA and 28.3%) ($P>0.05$). Only one pregnancy was observed in early and confirmatory diagnosis, result of a Control group embryo transfer. Pregnancy rates for Control group, FBS+CLA and CLA were, respectively, 4.35% (1/23), 0% (0/20) and 0% (0/28). Regarding the reexpansion, CLA treatment (47.1%) had lower rate ($P<0.05$) to Control (70.4%) and higher than the CLA+FBS (43.3%). The three treatments had similar hatching rates ($P>0.05$) by looking up 42.1%; 23.1%; 25% for Control; FBS+CLA; CLA groups, respectively. New embryo transfers should be carried out to evaluate the effect of CLA on in vivo embryo survival, endorsing the in vitro obtained reexpansion and hatching rates.

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Effect of handmade biopsy on bovine embryos at the sixth day post *in vitro* fertilization

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Keywords: biopsy, embryos, in vitro.

The genomic selection is a useful tool for animal breeding and it can be applied for in vitro produced bovine embryos through the analysis of embryo cells. However, it is necessary to evaluate the viability of the embryos biopsied at early stages. The aim of this work was to evaluate the impact of the biopsy technique without micromanipulation devices on development and further quality of embryos at the sixth day after in vitro fertilization. Oocytes obtained from ovaries collected at slaughterhouse were in vitro matured and fertilized. Followed in vitro fertilization, the presumptive zygotes were denuded and cultured in CR2aa medium supplemented with 2.5% SFB at 38.5ºC in an humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2. Embryos (morulas and early blastocysts) at the sixth day post-fertilization (D6) were randomly distributed in two groups: G1-control (n=61) and G2-biopsy (n=64). Embryos in drops of 20 L of TALP-HEPES with 2% SFB were sectioned by hand using microblades (Bioniche, Canada) under stereoscope microscope. After that, single embryos were cultured in drops of 20 L CR2aa. The blastocyst rate was evaluated on day seven (D7) and day eight (D8) post fertilization (24h and 48h after biopsy, respectively). On D8, the blastocysts from both groups were fixed and available by TUNEL assay (Promega, USA) for total cell number and number of apoptotic cells and then calculated the apoptotic index. The embryo development (blastocyst rate at D7 and D8) was analyzed by Qui-square. The total cell number, number of apoptotic cells and apoptotic index were analyzed by the t-Student test and their values are shown as mean +SEM. When only biopsied embryos at early blastocyst stage on D6 were analyzed, the blastocyst rate in G2 was lower ($P<0.01$) on D7 (43.5%) and on D8 (30.7%) than in G1 (78.7% and 67.8% on D7 and D8, respectively) but when the data of morula and blastocysts were grouped, the effect of biopsy on embryo viability was only perceived ($P<0.05$) on D8 (78.5% vs 43.5% for G1 and G2, respectively). There was no difference ($P>0.05$) in the total cell number between G1 and G2. However, the number of apoptotic cells was higher ($P<0.05$) in G1 than in G2 (29.4±4.5 vs 10.0±1.5, respectively). Similar finding was observed for the apoptotic index (27.5±3.9 vs 9.7±1.3, for G1 and G2, respectively, $P<0.01$). We concluded that the handmade biopsy in embryos on D6 influences the embryo development, but it doesn't affect the total cell number in blastocysts on D8, i.e, 48h post-biopsy. The lowest apoptotic index found in biopsied embryos may be due to the unnoticed withdrawal of cells from the inner cell mass during the biopsy, since this site display great number of apoptotic cells.

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Effect of protein source during culture on *in vitro* bovine embryo resistance to cryopreservation by slow freezing

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Keywords: BSA, classic freezing, feasibility.

The culture conditions can influence directly the quality of IVP embryos and their sensitivity to cryopreservation. Supplementation of IVM and IVC media with FBS is routinely used in IVP, however the presence of serum may result in accumulation of lipids and reduction in cry tolerance. BSA is an alternative source of protein that can be used to replace FBS that can improve the response of IVP embryos to cryopreservation (HIROYUKI ABE. MOLECULAR REPRODUCTION AND DEVELOPMENT.61:57-66.2002). The objective of this study was to evaluate the effect of these different protein sources on embryonic development and resistance to cryopreservation by slow freezing of bovine embryos. COCs obtained from slaughterhouse ovaries were selected and distributed into 2 groups: 1) control: COCs matured and cultured in media supplemented with 10 and 5% FBS, respectively; 2) COCs matured and cultured in media supplemented with 0.4% BSA. In D7, embryos at the BX stage from each group were divided into two treatments, half was kept fresh and the other half was frozen. The freezing was done using EG 1.5M in 0.25 ml straws and placed in Freezing Machine(FREEZE CONTROL® ,Model CL-863 System, Cryologic, Australia). After thawing the embryos were evaluated at 24 and 48 hours for reexpansion and hatching rates. Data were analyzed by chi-square test ($P < 0.05$). Cleavage rate was similar between group 1 (85.8%, n = 239) and 2 (81.7%, n=213), but the blastocyst rate was highest in group 1 (41.4%, n = 99 vs 26.8%, n = 57After thawing, the group cultured in the presence of FBS and cryopreserved, showed a lower hatching rate at 24 h (3.2%) than the fresh group (30.6%). While in the group cultured with BSA the hatching rate was similar between fresh (19.0%) and cryopreserved (13%) embryos. At 48 h of post thawing culture the hatching rate was lower in frozen than fresh embryos from both groups with FBS (6.5 and 61.1%) and with BSA (30.4 and 61.9%). However, for the cryopreserved embryos, a higher hatching rate was observed for those cultured in the presence of BSA than those in the presence of FBS. The results suggest that embryos produced in the absence of FBS although have lower blastocysts production have better resistance to freezing than those in the presence of FBS.

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Effect of supplementation with linoleic acid (omega 6) during *in vitro* production of bovine embryos on the modulation of intracytoplasmic lipid content and resistance to cryopreservation

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Keywords: bovine embryo, cryotolerance, linoleic acid.

This study aimed to evaluate the effects of the addition of linoleic acid (LA) to the culture medium in different moments of in vitro production (IVM, IVC or IVM+IVC) on the accumulation of intracytoplasmic lipids, development and cryotolerance of bovine embryos. COC's (n=526) were matured in TCM 199 with 25 mM bicarbonate, 10% FCS, 0.5 µg/mL FSH and 100 IU/mL hCG, supplemented or not with 100 mM LA, without mineral oil, for 22h at 38.5°C and 5% CO_2 in air. After, the oocytes were subjected to IVF and presumptive zygotes were cultured in SOFaa supplemented or not with 100 mM LA, without mineral oil, at 38.5°C and 5% CO_2 in air. Thus, there were four treatments: Control, LA during maturation (IVM), LA during embryo culture (IVC), and LA during IVM and IVC (IVM+IVC). The embryo development was evaluated on D7, when expanded blastocysts (Bx) were stained or cryopreserved. For determination of intracytoplasmic lipid content, a sample of Bx (n=133) was stained with the lipophilic dye Sudan Black B and stained embryos were evaluated under a light microscope running Q-Capture Pro Image software. The images were converted to grayscale and embryos were delimited to determine the lipid content. The Control group was chosen as the calibrator and the gray intensity value of each group was divided by the average of the calibrator to generate the values in arbitrary units. Other Bx (n=68) were vitrified by Vitri Ingá® (Maringa, PR, Brazil) method. The rates of re-expansion were evaluated immediately after warming (0h) and after additional 24h of IVC in SOFaa medium. The embryonic development rates and re-expansion rates were evaluated by Chi-Square, while the lipid content was subjected to analysis of variance (ANOVA) followed by Tukey's test ($P<0.05$). There was no significant difference ($P>0.05$) in the blastocyst rates between the Control (46.7%) and IVM (42.9%) and IVC groups (46.6%), but embryo production was lower ($P<0.05$) in IVM+IVC group (32.8%) compared to Control and IVC groups. The intracytoplasmic lipid content in embryos of the Control group (1.0±0.0) was similar ($P>0.05$) to that of the IVM group (0.9±0.1), but differed ($P<0.05$) from IVC (08±0.1) and IVM+IVC groups (0.7±0.1). Based on these results, we decided to evaluate the cryotolerance of embryos treated with LA only during IVC, and we observed that although no significant difference was found ($P>0.05$), the rates of re-expansion after 24h were numerically higher in IVC group (28/43; 65.1%) when compared to Control (18/39, 46.52%). Nevertheless, the biological effect was evident, since a greater number of transferable structures were retrieved. In conclusion, treatment with LA reduced the amount of intracytoplasmic lipids in bovine IVP embryos, which resulted in the recovery of a greater number of viable blastocyst after warming, but this difference was not statistically significant.

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Effect of two FSH ovarian stimulation treatments on doppler velocimetry and ovarian artery diameter in goats

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Keywords: color Doppler, goats, ovarian artery.

The aim of this study was to evaluate the effect of two FSH ovarian stimulation treatments on Doppler velocimetry and diameter of ovarian artery. 4 undefined breed goats 4-7 years of age were used. For synchronization of the cycle, intravaginal sponges were inserted containing 60 mg of MAP for 10 days, on the seventh day of treatment 50 ug D-cloprostenol was applied. For ovarian stimulation, the goats were divided into two groups; i) multi-dose (MD) with administration of 120 mg of pFSH intramuscularly, divided into five decreasing doses. Progestogen was administered on the 7th day of the treatment till 24 hours before sponge removal in 12 h intervals; ii) one-shot (OS) pFSH with 70 mg plus 200 IU eCG administered intramuscularly 36 hours before withdrawal of the sponge. Doppler ultrasound scanner apparatus (CTS-8800V, SIUI, China) equipped with linear transrectal probe (6-8 MHz) was used. Goats were maintained in stationed position and urinary bladder was used as a guide, the probe was inserted with the transducer perpendicular to the ventral abdominal wall and scanning was performed at 90° clockwise and 180° counterclockwise for location of the left and right ovaries, ovarian arteries and uterine veins. The examinations were performed before insertion and after removal of sponge to measure the diameter of vascularization in ovarian artery. The angle of insonation was set at 60, and the images were analyzed using the program Image J (National Institutes of Health, USA) previously calibrated (62.3 pixels/cm). Data was presented as mean ± standard deviation and paired-t test was used to assess differences between groups. The visualization of blood flow in ovarian artery in all ultrasound examinations was possible. The End Diastolic Flow (EDF) in the MD and OS group on D0 was 11.20± 1.94 and 13.5± 3.91, respectively. The average EDF on D10 in MD and OS groups was 15.3± 3.91 and 15.5± 5.17 cm/sec, respectively. The average Peak Systolic Velocity (PSV) in MD and OS groups on D0 was 25.6 ± 7.60 and 4.6± 28.5 cm/s, respectively, the same parameter on D10 averaged 27.4± 3.71 for MD group and 29.7± 6.08 cm/s for OS group, with no differences between groups in Doppler parameters ($P < 0.05$). The diameter on D0 of the ovarian artery in MD and OS groups was 0.21 ± 0.03 and 00.7 ± 0.17, respectively, whereas at D10, the averages were 0.30 ± 0.04 and 0.22 ± 0.03 in MD and OS groups, respectively. Significant differences ($P > 0.05$) were observed between days D0 and D10 in the MD group, but there was no difference between groups on D10. In conclusion, the use of two different treatments for ovarian stimulation did not alter the blood irrigation in caprine ovary.

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Effect of Fibroblast Growth Factor-10 during pre-IVM and IVM on quality of IVP bovine embryos

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Keywords: embryos, gene expression, oocyte competence.

Aiming to improve oocyte competence, a pre-IVM period in which the resumption of meiosis is blocked immediately after the oocyte retrieval has been proposed by several authors. However, no increase on embryos production has been obtained to date (Guemra et al., 2014, Theriogenology, v. 81, p. 982–7; Guimarães et al., 2015 Theriogenology, 83:52-7). Fibroblast growth factors (FGF) have an important role from since the beginning of oocyte formation until atresia or ovulation. Among the FGF family, addition of FGF-10 to the medium during IVM improves embryo quality (Zhang et al, 2010, Reproduction, v. 140, p. 815–26). This study aimed to evaluate if supplementation of the pre-IVM and IVM medium with FGF10 would improve the quality of bovine embryos assessed by the kinetics of development and the gene expression. COCs were aspirated from slaughterhouse ovaries and after selection, were divided into 3 groups: T1 (control), with COCs matured for 22 hours (N = 116); T2 (PMC-MC), with COCs pre-matured for 22 hours and matured for 22 hours (N = 119); T3 (PFGF-MFGF) with COCs pre-matured with FGF10 for 22h and matured for 22 hours (N = 112). The pre-IVM medium contained 0.2% BSA, 10-4UI / ml FSHrh and 10μM of cilostamide, and the IVM medium, 0.4% BSA and 10-1UI / ml FSHrh. The FGF10 concentration was 0.5 ng/ml. After maturation, COCs were fertilized and cultured until D8 of development. Hatched blastocysts on D8 were frozen in RNA Later for gene expression analysis by qPCR. Blastocyst rate was evaluated on D6, D7 and D8 and embryos were categorized into Bi, Bl, Bx and BE. The relative expression of KRT8, PAGE2, PLAC8, MSH6, HSPB1 genes, was assessed by qPCR using Fast Sybr Green Master Mix (Applied Biosystems). The ACTB gene was used as endogenous control. Data of blastocyst rate and kinetics of development were evaluated by chi-square test. The relative expression of each gene was calculated using the ΔΔCt method with efficiency correction by Pfaffl method, the treatments were compared using the Dunnett test ($P < 0.05$). There was no difference between treatments in the blastocyst rate at D6 and D7 (T1: 33.6; 53.4; T2: 28.6; 43.7; T3: 33.9; 47.3). On D8, T2 had lower blastocyst rate than the other groups. The addition of FGF-10 did not increase the rate of embryo development, since Bx rate on D7 and Be rate on D8 (T3: 64.2 and 72.0) was similar to T2 (50 and 64.6) and T1 (77.4 and 56.2). From all genes evaluated only MSH6, which is related to DNA repair, showed difference in expression between treatments, being lower in T3 group compared to the control ($P < 0.05$). The results suggest that the addition of FGF10 during pre-IVM and IVM does not affect the quantity or quality of bovine IVP embryos.

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Effects of Forskolin during *in vitro* culture on the modulation of intracytoplasmic lipid content and resistance of bovine embryos to cryopreservation

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Keywords: cryotolerance, Forskolin, in vitro bovine embryos production.

This study aimed to evaluate the effects of different concentrations of Forskolin, added to the *in vitro* culture medium, on the intracytoplasmic lipid content, development and cryotolerance of bovine embryos. COC's (n=848) were submitted IVM during 22h at 38.5°C and 5% CO_2 in air, in microdroplets of TCM 199 with 25 mM of bicarbonate, 10% FCS, 0.5 µg/mL FSH and 100 IU/mL hCG, covered with mineral oil. After, the oocytes were subjected to IVF and the presumptive zygotes were cultured in SOFaa (control Group), supplemented on D6 with 2.5 (F2.5 Group), 5.0 (F5 Group) or 10 µM of Forskolin (F10 Group), at 38.5°C and 5% CO_2 in air. The embryo development was evaluated on D7, when expanded blastocysts were stained or cryopreserved. For the determination of intracytoplasmic lipid content, a sample of expanded blastocysts (n=99) was stained with the lipophilic dye Sudan Black B and embryos were evaluated under a light microscope running Q-Capture Pro Image Software. The images were converted to grayscale and embryos were delimited for determination of lipid content. The control group was chosen as the calibrator and the gray intensity value of each group was divided by the average of the calibrator to generate the values in arbitrary units. Other expanded blastocysts (n=76) were vitrified by Vitri Ingá® - Maringa - PR - Brazil method. The re-expansion rates were evaluated immediately after devitrification (0h) and after 24 hours of in vitro culture in SOFaa medium. The embryo development rate and re-expansion were evaluated by Chi-Square test, while the lipid content was subjected to analysis of variance (ANOVA) followed by Tukey's test ($P<0.05$). There were no significant differences ($P>0.05$) in the blastocyst rates between the Control group (44.9%) and other treatments, but embryo production was lower ($P<0.05$) in F10 group (38.8%) compared to groups F2.5 (50.5%) and F5 (54.7%). The amount of intracytoplasmic lipids of the Control group (1.0±0.00) was similar ($P>0.05$) to F2.5 (0.9±0.03) and F10 groups (1.06±0.03), however it differed ($P<0.05$) from F5 group (0.8 ±0.1). Based on these results, F5 group was tested for cryotolerance and it was observed that the rate of re-expansion 24h was greater ($P<0.05$) in F5 group (72.2%) compared to the Control group (46.2%). In conclusion, treatment with Forskolin at a concentration of 5 µM is effective for reducing the amount of intracytoplasmic lipids and improves cryotolerance of IVP bovine embryos.

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Effect of cAMP modulators during oocyte *in vitro* maturation on nuclear maturation and cytoskeleton integrity of vitrified bovine oocytes

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Keywords: cilostamide, Forskolin, IBMX.

Cryopreservation of oocytes is a strategic tool in embryo IVP but with limited use due to the complex cellular structure of oocytes, being oocyte quality a factor that influences the success of the technique. In view of the role of IVM on oocyte quality, Simulated Physiological System Oocyte Maturation (SPOM; Albuz, Hum Reprod, vol 25, p 12; 2010), which utilizes cAMP modulators to achieve greater oocyte competence by the extension of meiosis block, was developed. The aim of this study was to investigate the effect of SPOM system on nuclear maturation and cytoskeletal integrity of vitrified bovine oocytes. Oocytes from slaughterhouse ovaries were divided into 8 groups: G1 (immature oocytes); G2 (matured in standard medium without FCS / 24 h); (G3 subjected to pre-IVM / 2 h in the presence of modulators of cAMP, Forskolin (100μM) and IBMX (500μM), and then the extended IVM / 28 h with Cilostamide (20μM) and FSH); G4 (immature oocytes vitrified and subjected to conventional IVM); G5 (immature vitrified and subjected to pre-IVM and extended IVM); G6 (submitted to pre-IVM, vitrified, and then the extended IVM); G7 (matured in commercial IVM - Bioklone ® Animal Reproduction, São Paulo, Brazil - with SFB / 24 h); G8 (immature vitrified and subject to commercial IVM, with SFB). For analysis of nuclear maturation, oocytes (n = 429 obtained in 3 replicates, 15 to 75 per experimental group) were stained with Hoechst 33324 for obtaining the rate of matured oocytes (MII). Cytoskeletal actin filaments were stained with Phalloidin Atto-532 to evaluate the actin staining patterns (Stained / Uninjured or not stained / Injured; n = 373 obtained in 2 replicates, 9 to 73 per experimental group). The M II rate was evaluated by chi-square test ($\chi 2$) and the percentage of staining patterns/integrity of actin by Fisher's exact test, in Instat GraphPad program, the significance level of 5%. The MII rate observed in groups was: G1 (Immature): 6.67 ^{a}; G2 (Standard IVM): 77.78 ^{c}; G3 (SPOM IVM): 76.19 ^{c}; G4 (VIT/Standard IVM): 31.43 ^{ab}; G5 (VIT/pré-IVM/extended IVM): 18.57 ^{a}; G6 (pre-IVM/VIT/extended IVM):): 39.47 ^{b}; G7 (Commercial IVM): 71,74 ^{c}; G8 (VIT/Commercial IVM): 25.71 ^{ab}. This result suggests that in vitrified groups maturation was impaired, but to a lesser extent in the group undergoing pre-MIV before vitrification. Regarding the pattern of actin (Uninjured / Injured), we observed: G1: 100.0^{a} / 0.0; G2: 100.0^{a} / 0.0; G3: 100.0^{a}/0.0; G4: 50.0^{bd} / 50.0; G5: 26.0 ^{c} / 74.; G6:60.3 ^{b} / 39.7; G7: 94,2^{a} / 5.8; G8: 37.9 ^{cd} / 62.1. Therefore, our results suggest that in vitrified groups the injured integrity pattern is predominant, except in the group undergoing pre-MIV before vitrification. The pre-IVM SPOM system favors meiotic progression and cytoskeletal integrity of oocytes undergoing vitrification, and its use can improve rates at oocyte cryopreservation programs.

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Interference of cGMP pathway on the expression of lipid metabolism transcripts in bovine cumulus-oocyte complexes matured *in vitro*

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Keywords: nucleotides, PDE5, sildenafil.

In adipocytes, lipolysis depends on the activity of cAMP or cGMP-dependent protein kinase (PKG), therefore, intracellular levels of cAMP and cGMP nucleotides regulate lipolysis; when elevated it is increased and when reduced lead to lipogenesis (LAFONTAN, Endocrinololy, 19 (4): 130-7.2008). Enzymes controlling cAMP and cGMP levels (synthesis and hydrolysis) or activated by them are expressed in oocytes (OO) and cumulus cells (CC) (Schwarz, Theriogenology, 81 (4): 556-64.2013), therefore, it is possible that manipulating these signaling pathways during maturation may influence lipid metabolism in cattle cumulus-oocyte complexes (COCs). The aim of this study was to investigate cyclic GMP pathway during oocyte maturation and evaluate its effect on the transcription of genes involved in lipid metabolism. Two experiments were conducted in which COCs were matured in TCM199 with 0.4% BSA or 10% FBS plus 10-5 M sildenafil (SDF- inhibitor of PDE5, the cGMP degrading enzyme) FBS+SDF+KT5823 (PKG inhibitor) for 22h. The control group matured with BSA only. After IVM, cGMP levels were measured (pool of 40 COC) using an immunoassay kit (Direct Cyclic GMP EIA) in experiment 1. In experiment 2, ATGL and PLIN2 (lipolysis related proteins) and CPT1B (beta-oxidation related protein) transcripts were assessed by real-time PCR in CC and OO from a pool of 20 COCs. Statistical analysis was performed by ANOVA followed by Tukey hoc test (SAS Institute Inc., Cary, NC, USA). The cGMP measurement data (three replicates), were transformed to log10 prior to analysis and for gene expression the values of $2^{-\Delta\Delta Ct}$ were considered (5 replicates). The SDF in groups matured COCs with BSA or FBS (56.84 and 53.08 fmol/pool) increased cGMP relative to control (38.07 fmol / pool, P>0.05) while SFB alone had the lowest values (23.08fMol / pool, P<0.05), but there was only difference between the group associated with KT5823 (61.47fMol / pool) in relation to that matured only with FBS (P <0.05). The three studied genes were expressed in both cell types, and ATGL has not yet been described in COCs. In CC, ATGL relative expression of SDF was reduced compared to the BSA and FBS (P0.05) and restored when associated with KT5823 (P<0.05) and CPT1B was elevated only with SDF+KT5823 (P<0.05). Regarding PLIN2, expression was increased by FCS compared to control BSA (P<0.05), but adding SDF returned to control levels and association with KT5823 increased expression (P<0.05), similar to FBS. In OO there was no effect of treatments (P<0.05). In conclusion: 1) PDE5 and PKG are involved in the control of cGMP levels in bovine COCs and SFB tends to reduce such levels; 2) the pathway studied influences the expression of genes involved in lipid metabolism in CC, which in turn may have an effect on lipid metabolism of oocytes.

Acknowledgments: FAPESP.

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Isolation of somatic cell derived from ear tissue of collared peccary (*Pecari tajacu linnaeus*, 1758) submitted to different vitrification techniques

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Keywords: cryopreservation, somatic cell, wild animals.

The cryopreservation of tissue samples from wildlife species, especially collared peccary (*Pecari tajacu*), is an interesting step in obtainment and conservation of somatic cells for use in nuclear transfer (cloning). However, tissue vitrification protocols need to be optimized to ensure maximum preservation of the viable characteristics of cells. Therefore, the aim of this study was to compare by *in vitro* culture (IVC) two ear tissue cryopreservation techniques [conventional vitrification directly, CVD, as Silvestre *et al*. (2004, Theriogenology 49, 221–229) or solid-surface vitrification, SSV, according to Carvalho *et al*. (2011, Theriogenology 76, 933–941)]. Thus, ear fragments (9.0 mm^3) of eight collared peccaries (4 males and 4 females) with 7-12 months old, from Center Multiplication of Wild Animals (CEMAS/UFERSA), were collected and cryopreserved in solution containing Dulbecco Modified Eagles Medium (DMEM) plus 3.0 M dimethylsulfoxide, 3.0 M ethylene glycol, 0.25 M sucrose and 10% fetal bovine serum. After two weeks, fragments warmed and not cryopreserved (control) were cultured *in vitro* and assessed for cell morphology, adhesion, early subconfluence (70% of the plate covered by cells) and cell viability by trypan blue (in %). All data from eight animals were analyzed by ANOVA followed by the appropriate *post-hoc* test. After collection of tissue samples, a total of 96 fragments were distributed for the three experimental groups (CVD: 32; SSV: 32 and control: 32 fragments). Of these, only one fragment of control group did not promote adherence after the IVC. All fragments adhered in all groups were able to cell confluence, with cell proliferation from days 7°, 5° and 3° for the CVD, SSV and control groups, respectively. Differences in initiation of proliferation were observed between the cryopreserved and control groups ($P<0.05$). In general, all cells showed fusiform features with oval nucleus in the center, suitable fibroblasts. Moreover, all the adhered samples were able to achieve subconfluence on days 17.5°; 18.3° and 18.0° to the CVD groups, SSV and control, respectively ($P>0.05$). No difference was observed for the viability of growing cells (CVD: 89% *vs.* SSV: 86% *vs.* control: 91%, $P>0.05$). Additionally, whereas the control group reached as maximum viability, cells derived from the CVD and SSV groups showed a viability of 98% and 95%, respectively. In conclusion, both vitrification techniques may be used for the cryopreservation of somatic tissue of collared peccary, allowing the isolation of viable cells for the cloning and genetic conservation of this species.

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Global methylation and hydroxymethylation patterns of bovine tissues

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Keywords: *Bos taurus indicus*, hydroxymethylation, methylation.

The ratio of 5-mC to 5-hmC seems to have an important role in epigenetic reprogramming during gametogenesis and initial embryogenesis, involved in gene expression regulation. The aim of this study was characterize the global patterns of DNA methylation and hydroxymethylation in *Bos taurus indicus*. Fetal tissues of newborn Nellore calves and Gyr bull's spermatozoa from epididymis (head an tail) or from ejaculate, were used as DNA source. Blood samples (n=19) e placental tissues (cotyledon and allantoidal membrane; n=12) were collected soon after birth, stored at -20ºC and DNA was extracted using the *QIAamp DNA Blood Mini* kit (QIAGEN, Hilden, Germany). Ejaculated spermatozoa were obtained through eletroejaculation (n=5) and spermatozoa of epididymis head (n=5) and tail (n=2) were obtained using the extravasation method. The DNA extraction was procedure using a *Salting Out* protocol. Global DNA methylation and hydroxymethylation were quantified using the *MethylFash Methylated* and *MethylFash Hydroxymethylated DNA Quantification* (Epigentek, NY, USA) kits, respectively. DNA methylation means in blood, allantoidal membrane and cotyledon were 12.68±6.75%, 17.26±15.07% and 12.94±5.49%, respectively. DNA hydroxymethylation means were 3.47±1.93%, 2.86±3.16% and 2.43±2.12%, respectively. It was not observed any difference between the evaluated tissues, but a difference in hydroxymethylation was observed in cotyledon between male and female (4.15±1.67% and 0.99±1.13%, respectively). DNA methylation means in spermatozoa of epididymis head and tail and ejaculated spermatozoa were 43.27±6.84%, 34.66±8.04% and 35.12±6.40%, respectively; the means of DNA hydroxymethylation were 13.69±7.22%, 6.71±3.03% and 14.34±2.79%, respectively. The 5-mC and 5-hmC patterns do not alter during the maturation and post-ejaculation processes. However, in spermatic cells, the methylation and hydroxymethylation levels were higher than in blood and placental tissues; an exception is the 5-hmC in epididymis tail spermatozoa, which did not present any difference to the other evaluated tissues. 5-mC/5-hmC ratios for blood, allantoidal membrane, cotyledon, and spermatozoa from epididymis head and tail and from ejaculate were 4.75±3.77; 10.41±10.13; 8.36±8.27; 4.31±2.91; 5.44±1.25 e 2.53±0.65, respectively, and they did not alter in different studied tissues. The results showed that 5-mC and 5-hmC levels alter among the different tissues evaluated and according the gender in extra-embryonic tissues. It is important to emphasize the pioneering of this work in bovine and the relevance of this knowledge to improve our comprehension about the epigenetic mechanisms.

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Short-term preservation of bovine COC in rainy season of Araguaína-TO: effects of temperature and time

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Keywords: bovine, conservation, COC.

Recent research has created extraordinary opportunities for animal reproduction, provided a major revolution in the multiplication of high economic potential animals. Biotechniques have been developed to optimize and support the other already established, such as IVF and MOIFOPA. Therefore, this study aims to evaluate the effect of time and transport of slaughtered bovine ovaries temperature on the Cumulus Complex - oophorus (COC) and analyze its viability after this procedure. For this purpose were collected 30 pairs of ovaries in the municipal slaughterhouse Araguaína, in rainy season, transported in thermos bottles containing 0.9% saline solution. For analysis, the ovaries were divided into six experimental groups, according to temperature and time each was analyze. The time intervals tested were 3, 12 and 24 hours at temperatures of 4°C and 37°C. Antral follicles were aspirated to obtain COC with syringe needles coupled to 25x7 or 25x8. After the follicular aspiration, COC obtained were stained with 4% Trypan Blue (1: 1) to verify the integrity of the plasma membrane of granulosa cells and / or oocyte. Descriptive statistics were performed and the results were expressed in percentage (Andrade et al. J. Small Rumin. Res. V. 43, p. 235, 2002). COC 281 bovine were evaluated, in which the ovaries were transported within three hours, at both temperatures, they do not have their COC stained with Trypan Blue, suggesting no interference with viability. However, the ovaries were transported for a period of 12 hours, they started to show a decline in viability of COC and when exposed to different temperatures, those transported at 4°C showed better retention rate (65%) when compared having a temperature of 37°C, which obtained a relatively low rate of viable COC (46%), suggesting damage to the plasma membrane of these cells. When tested 24 hours, ovaries, transported at 4°C presented with normal aspect, although only 13 COC were obtained, with 77% viability. Already the ovaries transported in 37ºC in that same period, were damaged and difficult aspiration, and showed 100% COC unviable. From these results, it was concluded that there is a decline in viability of bovine COC when the ovaries were transported in saline at 4°C or 37°C, for periods over 12 hours, during rainy season.

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Bovine embryo production by metaphase plate transfer

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Keywords: metaphase plate, oocyte competence, oocytes.

The metaphase plate transfer (MPT) between oocytes provides the ability to solve problems that can compromise the success of some assisted reproductive techniques. Among those, the age of the donor, the presence of abnormalities caused by mutation of the mtDNA and cytoplasmic damage due to cryopreservation can be mentioned. In addition, this procedure is a tool to study various aspects of the nuclear-cytoplasmic interaction in oocytes. The objective of this study was to evaluate embryo production from MII oocytes that had metaphase plate transferred to the cytoplasm of another enucleated oocyte. Cumulus oocyte complex (COC's) grades 1 and 2 obtained from slaughterhouse ovaries were matured for 20 hours at 38.5°C and 5% CO_2. After maturation, the COC's were denuded in 0.2% hyaluronidase solution and selected for the presence of polar body (PB) and cytoplasm homogeneity. The selected oocytes were divided into two groups: 1) control group: submitted to parthenogenetic activation (PA); 2) oocytes undergoing MPT and PA. Oocytes of group 2 were incubated in Cytochalasin D and Hoechst 33342 solutions in TCM-199, for 30 minutes. After incubation oocytes were enucleated with an aspiration pipette (diameter: 30 to 40 μm) coupled to a micromanipulator and immediately received a metaphase plate previously removed from a donor oocyte in MII stage. At the end of reconstruction, the structures were submitted to fusion (two pulses of 2.1 KV / cm). The fused structures (n = 42; 56.05% from total) and control oocytes (n = 42) were activated in Ionomycin 5 μM solution for 5 minutes and then maintained at 2 mM 6-DMAP solution for 4 hours. Subsequently, they were cultured for 7 days on cumulus cell (CC) monolayer at 38.5 °C and 5% CO_2. Presumptive zygotes were evaluated at D2 and D7 to cleavage and blastocyst rate, respectively. The results were analyzed by chi-square test with $P \leq 0.05$. The cleavage and blastocyst rates of group 2 (19.05% and 4.8%, respectively) were lower than the control group (90.48% and 47.6%, respectively). According to the results, we can conclude that MPT is a promising strategy to produce embryos from compromised competence oocytes due to freezing, by mtDNA alterations and/or other reasons.

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Embryo response from Piau and Moura porcine breeds to cryotop vitrification

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Keywords: embryo, porcine, vitrification.

The porcine breeds Piau and Moura are locally adapted and are being replaced by commercial breeds, which have increased the danger of being extinct. Therefore, there is a demand for conservation of genetic material of these animals. However, the cryopreservation of pig embryos has been limited by their high cryosensitivity. Vitrification and, specifically the Cryotop method, have been proposed as a more successful methodology for embryo cryopreservation for many mammalian species. This procedure has the advantages of no ice crystals formation and high cooling speed. In this study, we evaluated the response of pig embryos from Piau and Moura breeds to vitrification by Cryotop. Piau (n = 58) and Moura (n = 18) females had oestrus observed and on D6 after natural mating, in vivo embryo collections were performed by laparoscopic. A total of 353 embryos was recovered (108 Moura and 245 Piau) in different stages of development. For vitrification, only fresh embryos at BL and BX stages, grades I and II, were used. Embryos were distributed into control group (C) – not criopreserved, and vitrified group (V) for each breed. After the warming period, the embryos from both groups were cultured in SOF medium, at 38.5°C and 5% CO_2 in air. At 24 and 48 h of culture, re-expansion and hatching rates were evaluated. Hatched embryos at 48 h were frozen and stored for gene expression quantification by qPCR, in which SYBR Fast Green Master Mix was used. Data from re-expansion and hatching rates were analyzed by Chi-square test ($P< 0.05$) and the expression of genes was performed by $\Delta\Delta Ct$, corrected by Pfaffl, using the ACTB as an endogenous control. At 24 h of culture hatching rate was lower ($P<0.05$) in Piau embryos (57.4%) than in Moura embryos (83.9%), while for the vitrified hatching rate was similar ($P>0.05$) between Piau (8.9%) and Moura (0%). At 48 h of culture the hatching rate was similar ($P>0.05$) for the control embryos of both breeds (83.0% and 96.8%, respectively). The vitrified embryos of Piau and Moura breeds had similar ($P>0.05$) hatching rates (19.6% and 21.9%, respectively), but both presented lower ($P<0.05$) hatching rate than the control embryos. For gene expression analysis were used three embryos pools, C and V for Piau breed. Moura embryos were unable to perform qPCR, due to insufficient number. Among the analyzed genes, BCL2L1, BAK, CASP3, only BAX showed differential expression between groups, being more abundantly expressed for group V ($P = 0.04$) compared to C. The results indicate that porcine embryos, even vitrified by Cryotop, have low recovery after cryopreservation. However, the gene expression response suggests that embryos that hatched at 48 h reverted well from vitrification stress.

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Use of ethyleneglycol monomethyl ether as cryoprotectant in vitrification of IVP bovine embryos

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Keywords: cryoprotectants, ethylene glycol monomethyl ether, vitrification.

Research has been conducted to identify cryopreservation protocols using satisfactory vitrification process. However, little attention was given to the thermodynamic and chemical characteristics of cryoprotectants. The aim of this study was to determine the most effective concentration of ethylene glycol monomethyl ether cryoprotectant (EGMME) in the vitrification solution of in vitro produced (IVP) bovine embryos. This experiment determined the concentration of EGMME in the vitrification solution associated with better hatching rate after warming by measuring hatching rates and gene expression of 405 embryos, in 6 repetitions. The vitrification methodology was described previously by Vieira et al. (Animal Reproduction Science, v. 99, p. 377-383, 2007). On average it was used 22.5 embryos per treatment/repetition. Embryo hatching rate was obtained from the average value of each treatment; 30 warming embryos were transfer to synchronized recipients. The hatching rate of non-vitrified control group (63.8%) was higher ($p<0.05$) in comparison with the treatment of 20% EG and 20% DMSO (T2; 37.6%) and EGMME 20% DMSO and 20% (T3; 22.0%), which was similar ($p>0.05$) between each other. The hatching rate observed in the treatment EGMME containing 15% DMSO and 20% (T4; 10.3%) was lower ($p<0.05$) when compared with other groups. The gene expression of BAX (apoptosis promoter) and CCND2 (proliferation marker) did not differ ($p>0.05$) between groups, but the expression of Bcl-2 gene (inhibitor of apoptosis) was lower ($p<0.05$) in T4 compared with other treatments. Pregnancy rates at 30 days for T2 and T3 groups were both 26.6%. The embryos in T4 were not transferred to recipients. Therefore, EGMME can be used as cryoprotectant in vitrification solutions of IVP bovine embryos.

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Use of microfluidic in the analysis of profile of genes related to embryo quality from Nelore cows submitted to superstimulation with p36 or p36/eCG protocols

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Keywords: gene expression, microfluidic, ovarian superstimulation.

Superstimulatory protocols with exogenous gonadotropins have been widely used in bovine embryo technologies. Better understanding of follicular dynamics has enabled the development of hormonal protocols to boost fertility in cows, e.g. the P36 superstimulatory protocol. Studies have demonstrated that P36 adaptations, such as the replacement of the last two doses of FSH by eCG (P36/eCG protocol) improves embryo yield, possibly due to its capacity to stimulate both FSH and LH receptors, especially to FSH receptor. However, the quality of the embryos produced under the influence of these gonadotropins is conflicting, i.e. sometimes benefits and sometimes impairs embryonic development. Therefore, the present study aims to quantify the mRNA abundance of genes related to embryo quality in cows submitted to P36 and P36/eCG protocol. Nelore cows (Bos taurus indicus) were submitted to cycle synchronization (control group, n=20; non-superstimulated), P36 protocol (P36 group, n=15) and P36/eCG protocol (P36/eCG group, n=20). All animals were slaughtered 12 h after removal of progesterone device. Ovarian antral follicles from control group (3-8 mm) and superstimulated groups (> 9 mm) were aspirated for in vitro production of embryos. Total RNA was extracted from pools of 5 embryos (5, 3 and 5 pools for control, P36 and P36/eCG groups, respectively), followed by reverse transcription and pre-amplification in 14 cycles with Taqman® enzyme and Taqman® bovine-specific oligonucleotide primers. For quantification of the mRNA abundance of 46 genes related to embryo quality by RT-qPCR, we used the microfluidic platform BioMark HD System™ (Fluidigm®) with HX controller (96 samples and 96 assays at the same time, 96.96 Dynamic Array). The relative abundance was calculated by ΔΔCt method (target gene/GAPDH) with correction by Pffafl's equation. Superstimulatory effect was tested by ANOVA (parametric) or Wilcoxon test (non-parametric, statistical difference with $P < 0.05$). The mRNA abundance of IFITM3 and SOX2 was higher in blastocysts from control group compared to cows submitted to ovarian superstimulation, while the mRNA abundance of ACTB, AQP3, CASP3, CDH1, OTX2 and PAF1 was higher in blastocysts from superstimulated cows. Thus, the present findings demonstrate that the use of exogenous gonadotropins modifies the profile of mRNA abundance of genes related to embryo quality and it, therefore, might affect the competence of embryos generated from cows submitted to ovarian superstimulation.

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Vitrification of immature bovine cumulus-oocyte complexes: effects on embryo development and nuclear lamin

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Keywords: cattle, cryopreservation, cryoprotectant.

Compromised developmental competence after oocyte cryopreservation is due to damage in important structures. Nuclear lamins provides mechanical stability, nuclear shape and is involved in several processes such as DNA replication and repair. The aim of present study was to examine the effects of cryoprotectant exposure (experiment I) and vitrification by OPS (experiment II), of immature bovine Cumulus-Oocyte Complexes (COCs) on embryo development and structure of nuclear lamin. Ovaries were obtained from the local slaughterhouse. Only oocytes enclosed in a compact cumulus with an evenly granulated cytoplasm were selected for experiments. Base medium for preparation of cryoprotectant solutions was holding medium (HM), which consisted of HEPES-buffered TCM199 supplemented with 20% newborn calf serum. COCs were first exposed to HM for 3 min followed by first vitrification solution: 7.5% dimethylsulfoxide (DMSO) and 7.5% ethylene glycol (EG) in HM for 15 min. Then, oocytes were transferred to two drops of second vitrification solution: 16% DMSO, 16% EG and 1 M sucrose in HM for 20 s each. For warming, the oocytes were submerged for 1 min in warming solution 1 (HM plus 1 M sucrose) and transferred to warming solution 2 (HM containing 0.5 M sucrose) for 3 min and washed twice in HM for 5 min. Thereafter, COCs were processed for *in vitro* maturation, fertilization and culture. Samples of COCs were fixed and immunostained for oocyte nuclear lamin evaluation. For statistical analyze, Chi-square test was used to compare data among the experimental groups ($p<0.05$). Exposure of immature COCs exhibited lower blastocyst rates 29% (31/108) compared to the control 51% (101/199). Higher hatching rates were observed in control 22% (43/199) as compared to vitrified 6% (7/108). Organization of nuclear lamin in immature COCs exposed to cryoprotectants exhibited a higher proportion of contractions and morphological alterations (35%) compared to control group (0%, $p < 0.05$). The vitrification by OPS affected developmental competence showed higher of cleavage in control group 79% (203/256) than in vitrified group 8% (13/156). COCs of control group showed blastocyst development of 42% (107/256) whereas no blastocyst was observed in vitrified group. Nuclear lamins was strongly affected by vitrification in immature oocytes, exhibited higher proportion of alterations (72%) compared to control group (9%; $p < 0.05$). In conclusion, immature COCs seem to be better tolerant to the cryoprotectant exposure, however, vitrification result in low embryo development due, probably to negative effects on nuclear lamin.