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Systems biology analysis for the identification of biological processes and candidate genes for the prediction of oocyte donor cow quality for IVP

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The combination of OPU-IVP with modern genomic technology is foreseen to have a huge impact on cattle production. However, the complex biological mechanistic outcomes behind IVP are not fully understood. Systems biology provides a holistic view of the important biological mechanisms that control complex traits such as those related to IVP procedures. In this study, we applied a "Weighted Gene Co-expression Network Analysis" (WGCNA) to provide a better understanding of the biological mechanisms that control IVP performances and we used this information to identify candidate genes for the prediction of oocyte donor cow quality.

We sequenced total RNA from granulosa cells from aspirated oocytes collected from 23 individual slaughtered Holstein cows. A pool of oocytes from each animal was kept separately and used for IVP procedure to evaluate the performance of each donor cow, measured at blastocyst rate, kinetic score and morphology score. WGCNA of the RNA samples identified four groups of highly co-expressed genes called "modules" whose expression profiles were significantly correlated with the blastocyst rate (P-value <0.05). Functional analysis of the four modules highlighted a wide range of biological mechanisms (eg apoptosis, cell proliferation and development and oxidative stress) as well as important upstream regulators predicted to be activated (SMAD4, TP53, EGR1 and POR) or inhibited (TBX2, INSR and β-oestradiol),

The central genes in these modules as well as their upstream regulators are expected to be promising candidate genes to predict the oocyte quality of donor cows. These findings are expected to improve genomic-assisted IVP and to have a direct impact on cattle breeding.

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Development of a laparoscopic ovarian biopsy pick-up method for goats

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Biopsy pick-up (BPU) has been considered a safe method to harvest ovarian fragments from live animals for research and/or clinical purposes. However, no studies have been reported on the use of BPU to collect in vivo ovarian tissue in goats. The goals of this study were: (I) to test different biopsy needle sizes to collect ovarian tissue in situ using the BPU method (Experiment 1), and (II) to study ovarian tissue features such as preantral follicle density, morphology, class distribution, and stromal cell density in ovarian fragments obtained by BPU (Experiment 2). In Experiment 1, goat ovaries (n=20) were collected in a slaughterhouse and subjected to in situ BPU. Three needles (16, 18, and 20G) were tested. In Experiment 2, the more efficient biopsy needle from Experiment 1 (16G) was used to perform laparoscopic BPU in goats (n=8). After analgesia, three incisions were performed for the insertion of a laparoscope, atraumatic forceps, and the biopsy needle. At least one ovarian fragment was obtained per ovary and subjected to classical histology procedure. All statistical analyses were conducted using R software version 3.0.2. Spearman correlation test, chi-square test, Kruskal-Wallis test and Wilcoxon Mann-Whitney test were applied when convenient. Data are presented as mean ± SEM and percentages, and the statistical significance was defined as $P<0.05$. In Experiment 1, the recovery rate was greater ($P<0.05$) using 16G and 18G needles compered to 20G needle. The mean weight of ovarian fragments by the 16G needle's (1.5 ± 0.1 mg) was greater ($P<0.05$) than those collected using the 18G (1.0 ± 0.1 mg) and the 20G (0.9 ± 0.2 mg) needle's. In Experiment 2, 62 biopsy attempts were performed and 52 ovarian fragments were collected (90% success rate). Overall, 2,054 preantral follicles were recovered from 5,882 histological sections. Mean preantral follicular density was 28.4 ± 1.3 follicles per cm^2. The follicular density differed among animals and ovarian fragments within the same animal ($P<0.05$). In addition, according to the variance component analysis, the histological sections contributed to the greatest variability (70%) of the total variance of follicular density compared to ovarian fragment (29%). The mean stromal cell density in the ovarian fragments was 37.1 ± 0.5 cells per 2500 μm^2, and differed ($P<0.05$) among animals. Moreover, a positive association ($r = 0.18$; $P<0.001$) between preantral follicle density and stromal cell density was observed. The percentage of morphologically normal follicles was 70.1 ± 1.2, and differed ($P<0.05$) among animals. The majority (79%) of the morphologically normal follicles were classified as primordial follicles, and differed ($P<0.05$) among animals and within each ovary. In summary, a laparoscopic BPU method has been developed to harvest ovarian tissue in vivo with a satisfactory success rate in goats. Furthermore, as previously reported for other species, this study described, for the first time in goats, a high heterogeneity in follicular density, morphology, class distribution, and stromal cell density in ovarian tissue collected by BPU.

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The kinetics of first cleavages influences the energetic metabolism and the transcription pattern in bovine embryos produced *in vitro*

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The main goal of the present study was to evaluate how the kinetics of the first cleavages may influence the energetic metabolism and the transcription pattern in bovine embryos. For this purpose, embryos were produced in vitro following standard protocols. About 40 post insemination, embryos were evaluated for cleavage rate and classified as Fast (4 or more cells) and Slow (2 or 3 cells). Culture followed for 7 days in SOFaa medium at 38.5°C, 5% CO2 and saturated humidity. On D5, embryos were transferred to individual wells containing 20µL of the same medium. On D7, the medium was collected to quantify glucose, lactate and pyruvate consumption. The respective blastocysts were stained for the detection of reactive oxygen species (CellRox Green®, ThermoFisher Scientific – n=18) and evaluation of mitochondrial activity (MitoTracker Red CMXRos, ThermoFisher Scientific – n=18). Furthermore, blastocysts were also collected (24 embryos - 4 replicates) and submitted to mRNA extraction, cDNA synthesis and quantification of 96 transcripts of interest by RT-qPCR in the BioMark® HD system (Fluidigm, San Francisco). For statistical analysis, the variables were submitted to Kolgomorov-Smirnov normality test and later compared by Student t test. For gene transcription data, GAPDH was used as endogenous control for the calculation of ΔCt. Results show that cleavage rate is higher in Fast embryos (42.6% ± 5.3) than in Slow (26.7% ± 2.6). Similarly, conversion to blastocyst is also higher in the Fast group (40.9% ± 4.2 vs. 13.9% ± 3.6). Regarding the consumption of energetic substrates, we observed that Slow embryos consume more lactate (5.63 ± 0.50 vs. 7.82 ± 0.32, $p = 0.01$) and more pyruvate (6.18 ± 0), 16 vs. 6.79 ± 0.10, $p = 0.012$), but there was no difference in glucose consumption. In addition, Slow embryos also present higher mitochondrial activity ($p = 0.0009$) and higher amounts of ROS ($p = 0.029$). Finally, 24 of the 96 analyzed genes presented differences between groups ($p < 0.1$), with 22 genes having the highest number of transcripts in Slow (ACSL1, ADCY6, ATF4, BID15, CASP9, CAT, G6PD, GPX1, GPX4, GSK3A, HMO1, HSF1, HSP90AA1, IGFBP4, NFKB2, PFKP, POU5F1, PRDX3, RGS2 and TFAM) and only 2 genes in Fast group (Dnmt3a and SDHA). These genes are related to oxidative metabolism, stress response and cell death. The results show that although slow embryos possess a more active metabolism, they also have lower rates of development. These data corroborate Leese's (2002) "silent embryo" theory, which says that less viable embryos have a compromised genome, and therefore, their more "active" metabolism is reflected in the higher consumption of oxygen and nutrients, which are used by repair mechanisms. Thus, these findings indicate that the kinetics of the first cleavages influence the pattern of metabolism functioning in pre-implantation embryos.

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Inhibition of HSP90 associated to heat shock during *in vitro* maturation of bovine oocytes alters the relative amount of transcripts in 8-cell stage embryos

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HSP90 is a protein involved in cellular homeostasis and its inhibition during maturation reduces the oocyte developmental competence (Souza et al. 2015, Reprod. Fertil. Dev. 27:235). The present study investigated the relative amount of heat shock (*HSF1, HSP90, HSP40*) and totipotency (OCT4) transcripts in bovine embryos at 8-cell stage derived from oocytes exposed to an inhibitor of HSP90 (17AAG; 17-allylamino-17-demetoxigeldanamicina; Sigma, St Louis, EUA) associated to heat shock (HS) during *in vitro* maturation (IVM). Cumulus-oocyte complexes (COC) were allocated in four groups during IVM: Control - without both heat shock and 17AAG; HS - heat shock (41.5ºC) for the first 12h of IVM; 17AAG - 2µM of 17AAG for the first 12h of IVM, and 17AAG+HS - 2µM of 17AAG plus heat shock for the first 12h of IVM. *In vitro* maturation was performed in Nunc plates, containing 400µL of TCM199 medium (Invitrogen, Carlsbarg, USA) supplemented with porcine FSH (pFSH - Pluset, Lab. Callier, Espanha) and 10% estrus cow serum, and incubated under 5% CO_2, 95% humidity and 38.5ºC for 24h. The heat shock was performed under 7% CO_2, 95% humidity at 41.5°C. After maturation, oocytes were *in vitro* fertilized for 20h with $2x10^6$ spermatozoa/mL. The presumptive zygotes were cultured in four-wells plate with 500 µL of modified CR2aa medium supplemented with 2.5% FCS (Nutricell, Campinas, Brasil) in an incubator at 38.5ºC under 5% CO_2, 5% O_2, 90% N_2 and saturated humidity for 52h. Eight-cell stage embryos were washed three times in PBS plus 0.1% polyvinyl alcohol and then rapidly frozen in liquid nitrogen, and stored at -80°C. Three pools of 10 embryos per group where used for total RNA extraction with RNeasy Micro Kit (Quiagen,Valencia, CA, USA) and reverse transcribed using the SuperScript III First-Strand Synthesis Supermix (Invitrogen, Carlsbad, CA, USA). Relative quantification was performed by Comparative Ct quantification ($2^{-\Delta\Delta Ct}$) method relative to the sample with the highest delta Ct value in the control group (calibrator sample) and was based on primer efficiency. Analysis was performed by mixed model using the Proc Mixed command in the SAS 9.0 software. $P<0.052$ was considered significant and the relative amount values are presented as mean ± S.E.M. The relative amount of *HSF1* transcripts was higher ($p<0.052$) in 17AAG group than in Control and 17AAG+HS groups but similar to HS group. Higher ($p<0.03$) amount of *HSP90* transcripts was found in 17AAG+HS group than Control and HS groups, but similar to 17AAG group. No difference was found for *HSP40* and *OCT4* transcripts among groups. Those data show that despite inhibition of HSP90 during IVM can affect the expression of *HSF1* in 8-cells embryos, it does not have the same effect on expression of *HSP90*. In contrast, inhibition of HSP90 associated to heat shock influences the *HSP90* expression but has no effect on *HSF1* transcript. In conclusion, relative expression of genes in 8-cell stage embryos is influenced by the inhibition of HSP90 and heat shock during *in vitro* maturation of bovine oocytes. Financial support: CNPq, FAPEMIG and FAPES.

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Characterization of AMH gene polymorphisms and its association with traits indicative of sexual precocity in Nelore heifers

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Anti-Müllerian Hormone (AMH) is a protein expressed in the gonads and is related to ovarian follicular development. AMH plays an important role to prevent premature depletion of the follicular reserve, by promoting balance between inhibition and promotion of follicular growth and development. Recently, Pierucci et al. (2016; Abstract Book of 18th ICAR, p. 85-86) characterized the presence of polymorphisms in the coding region of the AMH gene in Nellore heifers. The authors identified four SNP-type polymorphisms; one located in intron 2 (rs132972253) and three in exon 5 (rs527023314, rs722016629 and rs134387246), the latter three being qualified as synonymous mutations. The aim of this study was to analyze the association between polymorphisms in the exon region of the AMH gene and early pregnancy occurrence (EPO) and age at first calving (AFC) traits in Nelore females, and evaluate the genetic constitution of this population for this gene. Phenotypic data consisted of the verification of conception or not conception of 197 unrelated heifers exposed in the breeding season, aging 16 to 18 months. Allelic and genotypic frequencies were calculated by allele counting. Hardy-Weinberg Equilibrium and Linkage Disequilibrium (LD) were calculated by Chi-square test and regression, respectively. The effect of the genotypes identified for the AMH gene in EPO and AFC, as well as the mechanism of action of these polymorphisms (additive effect, dominance or over dominance), considering a significance level of 10%, were analyzed using the GLIMMIX procedure of SAS 9.3®. For analysis of EPO, we considered a binomial distribution model and for AFC, a linear model with normal distribution was considered. All three SNP in the present study were under Hardy-Weinberg equilibrium. Low LD values (<0.3) were detected in all of the exon 5 SNP pairs, indicating independent segregation, despite the short distance between loci. Only SNP rs134387246, located in the stop codon, exhibited significant value for both phenotypes (EPO = 0.059; AFC = 0.060). The heterozygous individuals showed a shorter AFC (in 75 days) compared to the average AFC of the homozygotes, representing the condition of over dominance. The results of the present study indicate that the marker rs134387246 may be one among many loci that affect reproductive traits in a non-additive manner. For the first time in literature, polymorphisms of the AMH gene were associated with sexual precocity traits, wherein one of the SNP demonstrated a positive influence on them and could be further used as a tool for selection of Nelore females, allowing acceleration of genetic gain and efficiency improvement of cattle herds.

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Evaluation of L-carnitine supplementation on the production and vitrification of bovine embryos produced *in vitro*

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In vitro produced embryos (IVP) present low survival to conventional methods of cryopreservation. In order to improve production and survival rates after vitrification, L-carnitine (CA) was used in different doses and different cultivation moments of in vitro embryonic development. For this purpose, the oocytes IVM was performed in TCM199 medium supplemented with 25 mM of sodium bicarbonate, 1.0 µg/mL of FSH, 50 Ul/mL of hCG, 1.0 µg/mL of estradiol, 0.2 mM of sodium pyruvate, 83.4 µg/mL of amikacin and 10% of fetal bovine serum. After 24 hours of IVM, oocytes were co-incubated with semen in a Talp-IVF medium supplemented with 6 mg/mL of BSA for approximately 20 hours. The development culture (CIV) was performed in a SOFaa medium with 6 mg/mL of BSA and 2.5% of SFB. According to the design, CA was used in the concentrations of 0.0mM (control); 1.0 mM; 2.5 mM and 5.0 mM, from 96 or 144 hours after fertilization (hpf). All IVP cultures were performed in an incubator at 38.5 ºC and a CO2 atmosphere of 5% in air. At the seventh day, IVCs were evaluated for blastocysts rates, with the embryos being submitted to vitrification. The embryonic viability post-vitrification was evaluated by the re-expansion rate and embryos hatching after re-heating and cultivation for 48 hours within the same conditions of IVC. Embryos production had a completely randomized design with a factorial scheme 4 x 2 (four concentrations and two days) and four replicates, with data being transformed in arc sine and submitted to a variance analysis, with means compared by the Tukey's test at 1% probability, with the aid of the software SAS. Regarding categorical data analysis (expansion and hatching), the Chi-square test was used, considering the effects of concentration and day, as for P values equal or inferior to 0.01 ($p<0.01$), the differences between these effects were considered as significant. The moment of CA use did not interfere on blastocyst production ($P=0.3$), however the use of 2.5 mM resulted in a greater production of embryos (62.0±0.07%, $P=0.02$), followed by the groups 0.0 mM, 1.0 mM and 5.0 mM, respectively (53.6±0.02, 57.7±0.07 e 49.4±0.11, $P<0.05$). Both the embryonic re-expansion and hatching were influenced by the moment of application and the CA concentration. The use from 96 hpf (38.4 and 27.9) was better that 144 hpf (27.9 and 13.5), while the concentration of 2.5 mM resulted in a greater percentage of re-expanded and hatched embryos (37.7 and 27.0), when comparing to groups 0.0 mM, 1.0 mM and 5.0 mM, respectively (19.6 and 14.3; 22.8 and 15.7; 23.6 and 16.7 $P<0.05$). Considering the moment and concentration, the group 96 hpf/2.5 mM presented the greatest expansion rates of 60.7% ($P=0.008$) and hatching of 41.4% ($P=0.04$). Based on the obtained results, it was concluded that when using L-carnitine from 96 hpf with the concentration of 2.5 mM, the embryonic survival to vitrification is improved.

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Protein profile evaluation of bull sex sorted sperm

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Sperm sorting to obtain samples with high percentage of gametes carrying X or Y chromosomes makes relevant the role of artificial insemination, maximizing genetic progress. The identification of distinct proteins in the sperm membrane with X and Y chromosomes could allow the development of a technique for sperm immunosexing. Therefore, the objective of this study was to assess the protein profile of sex sorted sperm by flow cytometry. Sex sorted sperm (n=6X and n=6Y samples) were used. Proteins were extracted and analyzed in mass spectrometry by data independent acquisition. The data were searched against Swissprot and trEMBL database in Bos taurus taxonomy. For the variables analysis the data were normalized, transformed (log2) and compared using tools available in Perseus. The Student t test was used to compare the variables results. Significance level of $p \leq 0.05$ and fold change ≥ 1.5 were considered. There were 459 proteins common to both groups, 7 showed greater relative abundance between X and Y spermatozoa with fold change $>\pm 1.5$. The main proteins are FUN14 domain-containing protein 2 and NADH dehydrogenase [ubiquinone] iron-sulfur protein 7 mitochondrial are related to mitochondrial damage (MURRAY et al. J. Biol. Chem., v. 278, p. 13619-13622, 2003; CHEN et al. Autophagy, v. 12, p. 689-702, 2016) and their greater relative abundance in X may be related to lower sperm motility (BORO, Int. J. Appl. Res. v. 4, p. 460-462, 2016). In contrast the pyruvate dehydrogenase protein X component acts on the energy generation pathways, which was found in greater abundance in Y sperm and may be related to faster sperm motility (BORO, Int. J. Appl. Res. v. 4, p. 460-462, 2016). Similarly, predict dynein intermediate chain 2 axonemal is mostly responsible for sperm motility, and was too greater in Y sperm. Predict EF-hand domain-containing protein 1 is related to PLCζ activity, which produces calcium oscillation for embryonic development. Some outhors described that spermatozoa carrying the Y chromossome has greater embryonic growth rates (PERGAMENT et al. Hum Reprod, v. 9, p. 1730-1732, 1994). More studies are necessary to clarify the differential protein expression profile. Overall, the majority of proteins found was related to energy generation that leads an understanding of the greater motility in sperm carring Y chromossome. This study may be guidance for further experimental studies related to sex sorted sperm and a possible future development of the immunosexing.

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Characteristics of H3K4me3 and H3K9ME at different stages of development of bovine embryos produced *in vitro*

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Post-translational modifications in histones are involved in the regulation of gene expression during the embryogenesis process (Boland, M.J. et al., Circulation Research, 115, 311-324, 2014). H3K4me3 is involved in the permission of gene transcription (Liu X. et al., Nature, 537, 558-562, 2016), while H3K9me is involved in the repression of gene transcription (Santos F. et al., Current Biology, 13, 1116-1121, 2003). The aim of the present study was to characterize the modifications of global H3K9me and H3K4me3 during embryonic development in vitro. The embryos were produced in vitro and conditioned at different stages of development for the investigation of these histone. For the Immunofluorescence analysis, were utilized confocal microscopy (5 Pascal, Zeiss, Germany). The quantification of the fluorescence signal intensity of H3K4me3 and H3K9me were carried in 15 blastomers for embryos (with the exception of the 4 cels, in which all the blastomers were analyzed by ImageJ). Thy fluorescence signal in the z direction was corrected (pattern) and the date were normalized in relation to the negative controls. The Shapiro-Wilk test indicated the normality of the date. Scott-knott at 5% of probability was utilizded when a significant difference was identified in ANOVA test (Program R version 3.3.1). In all stages of embryonic development, twice proteins, H3K4me3 and H3K9me, were present, showing their importance in transcriptional control during 4 cell (higher intensity of global H3K9me) and hatched blastocysts (higher intensity of H3K4me3). These epigenetic marks appear to be related to the regulation of pluripotency. In the early blastocyst period, there is no significant difference in the behavior of the marks, which suggests a state of preparation of the embryos for a period of great modifications, the cellular differentiation. H3K4me3 and H3K9me are present among the stages of 4 cells and hatched blastocyst, however their relation is inverse, so that in the stage of 4 cells, H3K9me showed higher intensity and from the blastocyst phase the H3K4me3 showed higher signal intensity. The behavior of the H3K4me3 was more dynamic during the pre-implantation development than H3K9me, with peaks in the early blastocyst phase and the hatched blastocysts, demonstrating higher influence of the trimethylation of the H3K4 during the developmental of the blastocysts. The early blastocyst phase appears to be the transition phase between these modifications, since there not significant difference between the signal intensity of the both histone epigenetic modifications.

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Correlation between clarifide molecular markers and the final pregnancy rate of early heifers

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Molecular markers are a genomic tool, able to predict the reproductive potential of animals. The Nelore CLARIFIDE 2.0 uses the SNP Chip ZL5 with 25,458 markers for the Nelore breed. The objective of this study was to correlate the iMVPRep (bioeconomic reproductive index), with the final pregnancy rate of early heifers. The reproductive index simplifies the selection of multiple traits of maternal characteristics (cow productivity). Higher maternal index values indicate females or daughters with better reproductive performance and production characteristics. This index includes: age at first calving, probability of early calving, stayability, productivity gained, maternal ability at 120 days, weight at 210 days and weight at 365 days (Zoetis, Data on file, 2017). Were evaluated genomically (CLARIFIDE Nellore, Zoetis), 226 Nellore heifers, which after being weaned were submitted to feedlot supplementation for 150 days on a diet of 56% of bulky and 44% of concentrated for approximate consumption of 2.3% of body weight. Afterward they received supplementation with 3g/Kg of body weight until the end of the breeding season. All heifers, with an average age of 15 months, were exposed to the cycling induction protocol, previous to ovulation synchronization protocol and TAI. After insemination they were exposed to bulls on 1:30 ratio. The groups were divided by median according to the Rank iMVPRep. Group A (n = 116), 46% < in the Rank, animals with higher values of iMVPRep (158.83 ± 48.03); Group B (n = 107), 47% > in the Rank, animals with lower values of iMVPRep (32.11 ± 48.24). The heifers body weight at TAI day was adjusted to 450 days, being (297.7 ± 1.87) for Group A and (294.6 ± 1.87) for Group B, with no statistical difference (P = 0.23). The binomial variables (final pregnancy and TAI pregnancy rate) were analyzed by PROC GLIMMIX of SAS, with sire and technician as random variables, and the continuous variables (average weight adjusted to 450 days) were analyzed by PROC MIXED of SAS. A significant effect was considered when $P < 0.05$. There was an effect of reproductive index with the final pregnancy rate of heifers, 79.1% for Group A and 64.3% for Group B (P=0.04) and a trend in the TAI pregnancy rate, 49.2% for Group A and 35.4% for Group B (P=0.06). The animals identified by CLARIFIDE, with higher values of iMVPRep (greater reproductive potential), presented a higher TAI and final pregnancy rates comparing with animals with lower values of iMVPRep (lower reproductive potential), even with similar average body weight within groups. This data leads to a conclusion that heifers with greater iMVPRep had better reproductive performance and higher probability of early pregnancy. More studies are needed to elucidate the relationship between the parameters analyzed using genetic markers with reproductive performance (phenotypic) of animals.

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Effect of sex on survival of bovine *in vitro* produced embryos vitrified by Cryotop

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Several studies have shown that male and female embryos are different not only in speed of development, but also for metabolism, gene expression, epigenetic patterns and stress response. This study aimed to evaluate if the cryopreservation effects caused on embryos may vary between gender. Oocytes obtained from slaughterhouse ovaries underwent IVM for 24 hours, were inseminated with 1 x 10^6 spermatozoids/mL, co-incubated in IVF medium for 16-18 hours, and possible zygotes were cultured *in vitro* (IVC) for 8 days. Cleavage at D2 and blastocyst rates at D6, D7 and D8 were evaluated. At D7, grade I embryos at expanded blastocysts stage, according to IETS manual, were removed from IVC and divided in two treatments: control (C) and vitrified (V) by *Cryotop*. After warming process, embryos returned for additional 24 hours in ICV conditions, for survival (not degenerated embryos) and evolution rates evaluation. Afterwards, embryos from both treatments (C: n=129; V: n=165) were individually stored in DM-PBS with lysis buffer, at – 20 °C, for sex determination, that was assessed by polymerase chain reaction and confirmed in 1.5% agarose gel. Data were analyzed by Mann Whitney test ($P < 0.05$). Male (n=57 [44%] e n=89 [53.9%]) and female (n=72 [55.8%] e n=76 [46%]) embryos percentage were similar for both control group and vitrified one ($P > 0.05$), respectively. For the vitrified embryos difference between male and female data was not seen for survival rate (n=87 [55.1%] e n=71 [44.9%], respectively), evolution form expanded to hatched blastocyst rate (n=61 [52.4%] e n=50 [47.6%], respectively) and degeneration rate (n=2 [28.6%] e n=5 [71.4%], respectively). These results suggest that male and female embryos have the same vitrification tolerance for *Cryotop* method.

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Efficacy between two protocols for isolation of primary culture of equine uterine tuba cells

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In the last decade, the primary culture of uterine tube (CTU) cells has become an effective, rapid, economical and reliable way to obtain information about the BEHAVIOR and modulation of different components of tubal fluid in THE fertilization PROCESS, Clarifying the biology of this process. In this sense, it´s proposed to compare the effectiveness of CTU isolation by two protocols; Isolation with Scraped with histological lamina and catheter cannulation. Twenty ovaries of mares from slaughterhouses were collected according to the modified Nelis technique (2013). These were transported to the laboratory in a solution of PBS, penicillin and streptomycin (Penstrep®) and amphotericin (Fungizone®) at 4°C. The uterine tube (TU) was isolated and the portion between the tubal uterine union and up to two centimeters of the isthmus ampullary union was used. In group A (n = 10) cells were collected by scraping with light pressure from a histological slide over the entire length of the duct. The uterine tube (TU) was isolated and the portion between the tubo-uterine junction and up to two centimeters of the isthmic-ampulla junction was used. In group A (n = 10) cells were collected by scraping with light pressure from a histological slide over the entire length of the duct. In group B (n = 10) each tube was cannulated with a 24G catheter in the ampullary portion and approximately 500 μL of the previously mentioned solution was introduced by moderately massaging and then withdrawing all of its contentes. The fluid obtained from both techniques was centrifuged at 200xg for 10 minutes, the pellet formed was cultivated in 60mm Petri dishes containing TMC 199 medium (90%), fetal bovine sérum (10%), pen-strep (1.5%) and fungizone (1.2%). The medium was changed every 48 hours for a period of 4 days. In the preliminary analyzes, confluence, colony formation (explant), ciliary movement and amount of cell debris were evaluated. In group B, the amount of cell debris was lower, whereas in group A, confluence was better, suggesting that the extraction of epithelial cells with catheter aid significantly reduced the amount of cellular debris and the possibility of contamination, favoring the visualization and the management of explants. The ciliary movement, one of the most important characteristics, was present in both groups, and increased throughout the days. In the scraping technique, group A obtains a high amount of cell debris with a confluence of 50% in 48 hours. It is believed that the difference between the groups is due to the applied mechanical pressure, in order to give off more quantity when the force increases. Likewise, more aggressive techniques such as the use of enzymes to digest the tissue or scraping of the tubal epithelium can obtain more cells. According to the objective of the present work, it is believed that to obtain 100% viable explants with ciliary movement, it is necessary to cultivate for a time superior to 72 hours, contrasting with cattle, where the research group has observed with the same protocols viability and Ciliary movement with 24 hours of culture. It is concluded that the two methods are effective and can be used as validated protocols for obtaining equine explants. It is concluded that the two methods are effective and can be used as validated protocols for obtaining equine explants.

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PTGS2 Expression in cumulus cells is a potential biomarker of oocyte quality independently of patient's clinical variables

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Gene expression in Cumulus cells (CCs) have previously been suggested as a predictive tool for oocytes quality in several studies. Still, there is little consensus about which biomarkers would actually be clinically efficient and applicable (Fragouli, Human Reproduction Update, 20:1-11, 2014). We analyzed CCs gene expression data considering the patient's clinical characteristics and the oocyte's potential, with the aim to identify possible biomarkers of oocyte competence independently of patient's clinical characteristics. Pooled CCs samples were obtained from 29 patients submitted to ICSI procedure. The oocytes corresponding to the samples were accompanied until day 5 after ICSI, and samples were divided in Good Quality group (GQ) (n=11) and Poor Quality group (PQ) (n=18) accordingly to percentage of blastocyst formation (GQ= 50% or more of the sample's embryos generated blastocysts; PQ= less than 50% of the sample's embryos generated blastocysts). All embryos from the same patient were cultivated together. Each sample was submitted to reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) on StepOnePlus™ (Applied Biosystems, USA). Oligonucleotides were selected to be complementary to the human sequence of Anexin 1 (ANXA1), Prostaglandin-endoperoxide synthase 2 (PTGS2), Glutathione Peroxidase 4 (GPX4) and Glutathione-S-Transferase 1 (GST1). The relative expression was calculated by the 2-ΔΔCT method (Livak and Schmittgen, Methods, 25:402–408, 2001). Patient's clinical data (infertility diagnosis, BMI, age and stimulation protocols) and experimental data were combined using mice package (Buuren and Groothuis-Oudshoorn, Journal of Statistical Software, 45, 2011) in a multiple regression model built using the percent of blastocysts as dependent variable and clinical data as independent variables. Test models composed of clinical variables and each assay data were compared against the baseline model. All procedures and computations were performed in R statistical environment. Differences within the experimental groups were determined by Mann-Whitney test (GraphPad® Software 5.0). When submitted trough Mann-Whitney test, only ANXA1 expression, that has antiinflamatory properties, shown to be significantly different between GQ and PQ groups ($P<0.05$), being elevated in GQ group. Gene expression results were then submitted to linear regression analysis, which indicated that ANXA1 expression levels was not a potential predictor of oocyte quality when we considered clinical information. Contrarily, PTGS2 expression levels are overexpressed in GQ group ($P<0.05$), and this significance is independent of the clinical variables of each patient. As far as we are aware, this is the first study suggesting biomarkers found in CCs that predicts blastocyst formation potential regardless of patients age, diagnosis, BMI and stimulation protocol.

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Gene expression of VEGF in the corpus luteum of rats that ingested Black Tea (Camellia Sinensis (L.) Kuntze)

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Black tea is one of teas derived from the Camellia sinensis plant. Some studies determined that catechins of decreased the gene expression of Vegf (gene of vascular endothelial growth factor) in tumor, however the influence of black tea in the VEGF of the ovary have not been studied until now. The VEGF is an important angiogenic factor in the reproductive organs.This study aimed to verify the effects of intake of black tea on the relative abundance of Vegf mRNA in rats. For this purpose, the rats were divided into two groups, with 30 animals in each group: control group (CT), which received water, and black tea intake group (BT) ad libitum at the water bottle. Black tea was prepared daily at a concentration of 2.5% by the addition of boiling water in the pure extract of black tea and subsequently filtered, the animals consumed on average 27.56 mL/day of black tea.The ovaries were collected from 10 animals in each group at the end of every month, for three consecutive months, stored in TRIzol® (Thermo Fisher Scientific, California, USA) in a freezer at -80°C and the relative abundance of VEGF mRNA were subsequently evaluated for qPCR. Three endogenous genes were tested: beta-actin, Gapdh (glyceraldehyde-3-phosphate dehydrogenase), Hprt-1 (hypoxanthine-guanine phosphoribosyltransferase) and Rps-18 (18S ribosomal protein). Hprt-1 was the most stable gene and therefore was used as the normalizer of the reaction.The data were evaluated using the Mann-Whitneytest between groups at different collection moments ($p < 0.05$). The means and standard errors of relative expression of Vegf were: CT = 1.06±0.13 and CP = 2.09±0.18 (first month); CT = 1.08±0.14 and CP = 0.94±0.18 (second month); and CT = 1.05±0.12 and CP = 1.36±0.35 (third month). The relative abundance of mRNA of Vegf was higher in the group that consumed black tea in the first month of experiment ($p=0,0048$), in the other months no statistical difference was observed between groups. It wasconcluded that the black tea intake increases the Vegf expression in the corpus luteum of Wistar rats only in the beginning of the consumption, after that, the expression normalizes. It was concluded that the ingestion of black tea increases the expression of Vegf in the corpus luteum of Wistar rats only in the first month of consumption in relation to the control, in the following months the expression does not differ between the two groups. Apparently, the increase in the relative abundance of Vegf mRNA in the first month demonstrates a molecular regulation of the body to maintain the physiological ovarian angiogenesis, but further studies should be performed to prove the safety of black tea for female reproduction. FAPESP (2010/20274-0).

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Conceptus elongation in beef heifers with superior uterine capacity for pregnancy

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Infertility and subfertility represent major problems in domestic animals and humans. To select animals with intrinsic differences in pregnancy loss, beef heifers were subjected to serial embryo transfer and classified based on day 28 pregnancy rates as high fertile (HF=100%), subfertile (SF=25-33%), or infertile (IF=0%). Studies using in vivo-produced embryos established that preimplantation conceptus survival and growth to day 14 was not compromised in the SF and IF heifers (Geary, Biol Reprod, 95:47 2016). Thus, the observed difference in fertility was hypothesized to manifest during conceptus elongation and pregnancy recognition. Two in vivo-produced embryos were transferred into HF (n=21), SF (n=10), and IF (n=5) heifers on day 7. On day 17, the uterus was flushed and endometrium collected. Binary data were analyzed by logistic regression using the LOGISTIC procedure of SAS. Continuous data were analyzed by ANOVA using the GLM procedure or in a Poisson regression repeated measurements model using GENMOD procedure of SAS. Conceptus recovery rate was higher ($P<0.05$) in HF (71%) and SF (90%) than IF (20%) heifers. Interferon tau (IFNT) in the uterine flush was quantified by ELISA. Conceptus length was positively ($P<0.01$) correlated ($R=0.79$) with IFNT in the flush. IFNT was greater ($P<0.05$) in uterine flush from pregnant HF (148+54 ng/mL) compared to SF (74+67 ng/mL) and IF (0 ng/mL) heifers. Conceptuses from HF (x=10.6 cm, range=1.2-32.2 cm) were longer ($P<0.01$) than SF (x=4.7 cm, range=1.5-13.5 cm) or IF (<0.1 cm) heifers. Total RNA was sequenced (n=5 per group) from the day 17 endometria of open or nonpregnant (NP) HF, SF and IF heifers and pregnant (P) HF and SF heifers as well as 17 HF conceptuses and 10 SF conceptuses. RNA-Seq data was analyzed by EdgeR-robust analysis. There were 96 differentially expressed genes (DEGs; FDR $P<0.05$) in NP endometrium; several DEGs encoded proteins involved in immune responses or present in immune cells. Comparison of P and NP endometrium in HF or SF heifers found 3,422 and 1,095 DEGs, respectively, but no difference in IFNT-stimulated genes. There were only 168 DEGs in the endometrium of P HF and SF heifers, but the response to pregnancy was significantly diminished in SF heifers. There were 1,287 DEGs between HF and SF conceptuses. In contrast, only 3 DEGs were detected in long (9.8-32.2 cm) versus short (1.2-6.9 cm) HF conceptuses. Several transcripts encoding secreted proteins and involved in lipid metabolism were dysregulated in the SF as compared to HF conceptuses. Many of the down-regulated genes are associated with embryonic lethality in other species. These studies support the idea that the uterine environment directly affects conceptus survival and elongation during the establishment of pregnancy and asynchronous conceptus-endometrial interactions result in pregnancy loss after conceptus elongation during the implantation and embryogenesis phase of early pregnancy. Supported by NIH R01 HD072898.

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Identification of isoform PR-B in canine corpus luteum through the quantitative analysis of RNA sequencing (RNA-seq)

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Progesterone and its receptor are involved in the regulation of gene expression and can affect cell proliferation and differentiation in the canine corpus luteum (CL). The identification of progesterone receptor (PR) isoforms is fundamental to characterize the role that each isoform exerts in CL function. The objective of this study was to identify possible PR isoforms during non-gestational diestrus in the canine species, using the RNA-seq technique. For this purpose, the corpus luteum of ovaries from bitches that had undergone salpingo-hysterectomy were used on days 10, 20, 30, 40, 50, and 60 (n = 3/group) after ovulation. Preparation of the libraries and sequencing of the new generation followed the TruSeq RNA Sample Preparation Guide protocol described by Illumina. The reads were mapped against the reference genome (Canis_familiaris.CanFam3.1.75.dna.toplevel.fa) using the Hisat program ('Our Galaxy' - ETH-Zurich) which generates files in BAM format (Binary Alignment/Map). The sequencing data were statistically analyzed using the Cuffdiff program, a Cufflinks pipeline package, with the relative abundance of the transcripts measured in FPKM (Fragments per kilobase of exon per million fragments mapped). The BAM format files were indexed in IGV visualization software (Integrative Genomics Viewer) (Robinson et al.; Nat Biotechnol.; 29:24-26,2011), the reference genome being selected and the PR gene identified. From the alignment data generated in PR, it was possible to verify the presence and relative expression of the isoform by Sashimi plots, in which each exon junction is illustrated and the reads are enumerated. The Sashimi plot identified only the progesterone B receptor isoform (PR-B) in all periods, indicating a greater expression on days 10 and 20 after ovulation. This result was not expected, we also considered find the isoform of the progesterone receptor A (PR-A), since the secretion of the hormone progesterone reaches maximum values at this stage and the possibility of different isoforms was assumed. However, the result offers prospects for further studies on the role of the PR-B isoform in relation to proliferation and cell survival in the canine corpus luteum during diestrus.

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New insights into the proteomic abundance and the action of L-arginine during *in vitro* sperm capacitation of cattle

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The aim of this work was to evaluate the proteomic changes of bovine spermatozoa after heparin-induced *in vitro* sperm capacitation with the addition of L-arginine (L-arg) using the shotgun approach. It was also evaluated the sperm capacitation pattern, membrane integrity, mitochondrial activity, sperm motility and vigor to confirm the effect of L-arg during *in vitro* capacitation. The evaluations were respectively assessed by chlortetracycline staining, H342/PI, JC1, optical microscopy, and the proteomic abundance by nUPLC-MS/MS analysis. Sperm cells from three Nellore bulls (*Bos taurus indicus*) went through *in vitro* capacitation for 3 hours in sp-TALP medium supplemented with 20 μg/mL heparin (Control), or with 20 μg/mL heparin plus 1 mM L-arg (treatment). Data were subjected to analysis of variance (Proc GLM) and the averages compared by SNK test at 5% probability. When comparing to Control, the percentage of sperm motility was higher in the capacitation group treated with L-arg (67.50 ± 8.66% vs 55.00 ± 7.98%, $P < 0.05$) and there was an increase in the percentage of capacitated pattern (70.30 ± 1.85% vs 57.84 ± 2.15%, $P < 0.05$). After 3 hours of incubation, sperm capacitated with L-arg showed higher mitochondrial potential and showed increased membrane integrity comparing to Control (77.15 ± 5.02% vs 57.72 ± 3.13% and 57.15 ± 4.71% vs 42.85 ± 4.71%, $P < 0.05$). The proteomic approach identified 367 proteins in the bovine sperm after the *in vitro* capacitation. Forty were found to be differentially abundant between Control and treatment ($P < 0.05$), one was abundant only in the treatment with L-arg ($P < 0.05$) and 326 were unchanged ($P > 0.05$). Eleven proteins were upregulated and 29 were downregulated in treatment comparing to Control. In conclusion, the addition of L-arg to the culture medium in presence of heparin *in vitro* showed different protein abundance pattern, and increased the bovine sperm quality and the percentage of capacitated sperm. This proteomic change may be closely linked to the molecular mechanisms involved in the action of L-arg on the *in vitro* sperm capacitation of cattle. Further investigation should be performed to determine whether if these findings have any clinical value for fertility/infertility assessment.

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Transcripts levels of enzymes involved in histone acetylation in bovine oocytes of different competences

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Oocyte competence refers to the ability of an oocyte to undergo maturation, to be fertilized and to have normal embryonic development. Studies have reported the importance of enzymes involved in the acetylation of histones in oocyte during maturation and their possible association with oocyte competence. The aim of the present study was to analyze the expression profile of genes involved in histone acetylation and deacetylation in bovine oocytes of different competence levels in maturation. COCs were recovered from 1.0-3.0mm (less competent) and 6.0-8.0mm diameter follicles (more competent) dissected from the ovarian cortex. Oocytes from each group were matured in vitro for 0, 8 and 24 hours and stored for gene expression analysis. Total RNA was extracted from 4 pools of 15 oocytes, from each treatment at each maturation time. The levels of the gene transcripts involved in acetylation (HAT1, KAT2A) and histone deacetylation (HDAC1, HDAC3) were determined by qPCR, being the expression values normalized by the constitutive gene PPIA. Data were analyzed by ANOVA, and the means of each treatment were compared by Tukey test at the significance level $p < 0.1$. The results showed that the expression pattern of the genes studied was similar ($p > 0.1$) for more and less competent oocytes, not change during maturation. With the exception of the HAT1 gene, in which its transcripts increased ($p=0.05$) between 0 and 8 hours of maturation in the most competent group. When the different groups were compared at the same maturation time, the most competent group presented higher expression ($p=0.06$) of HAT1 and HDAC1 ($p=0.03$) at 8 hours of maturation than the less competent group. The other genes had similar expression in the different treatments ($p > 0.1$). It can be concluded that transcription of HAT1 gene had occurred during maturation in the most competent group, and that this group shows a improve expression of the HAT1 and HDAC1 genes at 8 hours of maturation than the less competent, suggesting that these can be used as markers for oocyte competence.
Support: Embrapa and FAPEMIG.

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The use of linoleic acid in *in vitro* culture of bovine embryos and its effects on production and survival to vitrification

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In the attempt to produce in vitro bovine embryos more resistant to vitrification and to maintain the embryo production levels, the trans-10; cis-12 conjugated linoleic acid (CLA t10, c12) was use in different dosages and different moments of culture. For this purpose, the oocytes IVM (in vitro maturation) was performed in TCM 199 medium supplemented with 25 mM of sodium bicarbonate, 1.0 µg/mL of FSH, 50 UI/mL of hCG, 1.0 µg/mL of estradiol, 0.2 mM of sodium pyruvate, 83.4 µg/mL of amikacin and 10% of bovine fetal serum. After 24 of IVM, oocytes were co-incubated with semen in a Talp-IVF medium supplemented with 6 mg/mL of BSA for approximately 20 hours. The in vitro culture (IVC) was performed in a SOFaa medium with 6 mg/mL of BSA and 2.5% of SFB. According to the experimental design, CLA t10, c12 concentrations were 0.0 µM (control); 50.0 µM; 100.0 µM and 150.0 µM from 96 or 144 hours after fertilization (hpf). All IVP (in vitro production) cultures were made in incubators at 38.5 ºC and CO2 atmosphere of 5% in air. At the seventh day of IVC the produced embryos indexes were evaluated, being the blastocysts of good quality submitted to vitrification. The embryo viability post-vitrification was evaluated by the re-expansion and hatch rate of embryos after re-heating and cultivation for 48 hours at the same IVC conditions. The embryo production had a completely randomized design with a factorial scheme 4 x 2 (four concentrations and two days) with six replicates and data were transformed in arc sine and submitted to a variance analysis, with means compared by the Tukey's test at 1% probability, with the aid of the software SAS. The Chi-square test was used for the analysis of categorical variables (expansion and hatching), considering the effects of concentration and day, as for P values equal or inferior to 0.01 ($p<0.01$), the differences between these effects were considered as significant. Were treated from 96 hpf 1564 zygotes, 0.0 µM (278); 50.0 µM (401); 100.0 µM (448), 150.0 µM (437); and from 144 hpf 1566 zygotes, 0.0 µM (278); 50.0 µM (469); 100.0 µM (441), 150.0 µM (378). Using CLA t10, c12 did not alter the in vitro production of embryos, regardless of dosages 0.0 µM (56.1±0.08), 50.0 µM (50.1±0.06); 100.0 µM (50.8±0.05) and 150.0 µM (50.1±0.08) or the moment of application 96 hpf (51.2±0.07) or 144 hpf (50.1±0.08) (P=0.4). The embryos' re-expansion and hatching after vitrification was positively influenced with the use of CLA t10, c12 with any dosage 50.0 µM (63.6 e 43.8%), 100.0 µM (64.6 e 39.6%) and 150.0 µM (70.3 e 53.3%) when comparing to the control group (34.7%; 16%) and to the start day of supplementation, with 96 hpf being more efficient than 144 hpf (70.4% and 50% vs. 48.2% and 28.4%, respectively), regardless of the used concentration (P=0.01). Considering both the moment and concentration, the group 96 hpf/150 µM presented the greatest expansion (85.5%) and hatching (70.9%) rates post-vitrification ($P<0.05$). Based on the results, it was concluded that when CLA t10, c12 is used from 96 hpf at the concentration of 150 µM, the embryo survival to vitrification is improved, without impairing production.

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Protein profile of ovarian follicular fluid in brown brocket deer (Mazama Gouazoubira): Preliminary results

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The brown brocket deer (Mazama gouazoubira) is a species of cervidae found in the Northeast Brazil. This species already presents difficulties with the changes of its habitat and antropic actions. Therefore, programs that seek the conservation of this species may benefit from reproductive techniques, such as the in vitro embryo production. Thus, it is interesting to carry out studies on the proteomics of follicular fluid in order to understand both development and maturation of oocytes. The aim of this study was to quantify the total proteins and to describe the fluid protein profile two follicular categories: I (< 3.5 mm) and II (> 3.6 mm). For this, four adult females were submitted to a hormonal treatment with progesterone, estradiol benzoate and equine chorionic gonadotrophin. Females were starved for 36 h and submitted to an inhalation anesthesia. Follicular aspiration was performed by videolaparoscopy and samples were centrifuged (3000xg for 20 min) individually according follicular category size. The supernatant was stocked -80°C for posterior use. Evaluation of protein concentration was performed by the Bradford method. Also, samples were submitted to SDS-PAGE electrophoresis, using a total of 15 µg proteins/well. Gels were stained with Comassie Blue G-250 and images were analyzed using Software Quantity One 4.5 (Bio Rad, USA). The main bands were submitted to tryptic digest and tandem mass spectrometry (ESI-Q-Tof) and identified using the MASCOT software. Statistical analysis was performed using Student t test ($P < 0.05$). No significant differences were observed for total amount of protein (mean ± sd) between category size: 49.20 ± 22.76 µg/µL (category I) vs 56.70 ± 27.38 µg/µL (category II). Analysis of gels showed the following distribution: 180-115 kDa (29%), 82-64 kDa (36%), 49-19 kDa (28%) and 15-6 kDa (7%). According to molecular weight, the major proteins were: haptoglobin, immunoglobulin gamma and complement C3 (180-115 kDa); ceruloplasmin X1, immunoglobulin G1, plasminogen, apolipoprotein A-1, alpha 1-β glycoprotein and serpine (82-64 kDa); serotransferrin, apolipoprotein A-1 and complement C3 (49-19 kDa) and hemoglobin subunits beta-3 and alpha-like (15-6 kDa). This study provides the first description of the proteome in brown brocket deer ovarian follicular fluid. Studies in this area should continue in an attempt to identify potential markers of oocyte quality in this species.

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Quantitative proteomic profiling of bovine follicular fluid during follicle development

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Follicular fluid (FF) constitutes the microenvironment of follicles and includes various biologically active proteins that can affect follicle growth and oocyte fertilization. The aims of this study were to investigate the proteome profile and functional overview of bovine FF during different stages of follicle development and to evaluate the association of the identified proteins with local steroids concentration. Eighteen healthy non-lactating Holstein cows were used in this experiment. Cows had their estrous cycles synchronized with Ovsynch added with an intravaginal progesterone device. Transrectal ultrasonography (MyLab30, Esaote, Genova, Italy) was performed every 12h to monitor the follicular dynamics. Follicles were individually aspirated at pre-deviation (F1~7.0mm); deviation (F1~8.5mm); post-deviation (F1~12.0mm); and pre-ovulatory stages of follicle development, which were confirmed by measurement of follicular estradiol and progesterone concentrations by ELISA. The FF from nine cows were selected for proteomic analysis. After albumin depletion, triplicates of pooled FF were reduced, alkylated, and digested with trypsin. The resulting peptides were labelled with TMTsixplex (Pierce, Rockford, USA) and quantified using LC-MS/MS (Orbitrap Elite, Thermo, San Jose, USA). Proteomic data were compared by PROC GLM of SAS (SAS Institute, Cary, USA) and associations between steroids concentrations and relative abundance of the differentially expressed proteins were tested by Pearson's correlation test. A total of 143 proteins was identified and assigned to a variety of biological processes, including biological regulation, response to stimulus, metabolic processes, defense response, and transport. Twenty-two differentially ($P<0.05$) expressed proteins were found between stages indicating intrafollicular changes over follicle development, with presumed deviation time critical to modulate the protein expression. Follistatin, inhibin, serglycin, spondin-1, fibrinogen, and anti-testosterone antibody were found to be relatively more abundant during early stages of follicular development. In contrast, apolipoprotein H, alpha-2-macroglobulin, plasminogen, antithrombin-III, and immunoglobulins were up-regulated after deviation. Amongst the differentially abundant proteins, 19 were found to be associated with steroidogenesis. Canonical pathways analysis using IPA software (Qiagen, Redwood City, USA) highlighted the occurrence in FF of functional networks in a differential temporal balance and control, including activation/inhibition of the acute phase response, coagulation system, complement system, liver/retinoid X receptor activation, and biosynthesis of nitric oxide and reactive oxygen species. The results provide new insights into the size-dependent protein changes in ovarian follicle microenvironment, associated with changes in local steroids concentrations, which are critical for follicle maturation and may influence follicular function.

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In vitro embryos production in bovine after metaphase plate nuclear transfer

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Studies have shown that damages of the cytoplasmic organization is one of the main alterations caused by vitrification in bovine oocytes, being one of factors responsible for the inefficiency of this technique. Genomic nuclear transfer (GNT), in which the DNA of a damaged oocyte is transferred to a viable oocyte cytoplasm with the aid of a micromanipulator, is an alternative to rescue genetic material from oocytes with compromised cytoplasm, such those that have been submitted to vitrification. The objective of this study was to evaluate the viability of the GNT technique in bovine oocytes. Two experiments were performed. In the first experiment, the ability of reconstructed structures to develop to blastocyst stage after Parthenogenetic activation (PA) was evaluated. The second experiment aimed to evaluate the capacity of the reconstructed structures to be fertilized and to determine if the sperm concentration could affect fertilization rate. For the first experiment, cumulus-oocyte complexes (COCs) obtained from slaughterhouse ovaries were matured for 21 hours and distributed into three groups: 1) previously enucleated cytoplasm (n=275) reconstructed with a metaphase plate from another oocyte (GNT-MP) and submitted PA; 2) PA control (n=141)and 3) IVP control (n=204). In the second experiment, COCs were matured, micromanipulated and divided into 3 groups: 1) GNT-MP fertilized with 1x106sptz / ml (n=64); 2) GNT-MP fertilized with 0.5x106sptz / ml (n=63); 3) Control IVP (n=92; fertilized with 1x106sptz / ml). After 18 hours of fertilization, the structures were denuded, fixed in acetic acid: alcohol (1: 3) for 48 hours and stained with lacmoid. Oocytes were then classified as fertilized, unfertilized, polyspermic and abnormal. The chi-square test was used for the rates of fertilization and blastocyst production considering the value of $P \leq 0.05$. In the first experiment, no difference was found between the control PA and control IVP groups for both cleavage (83% and 80.4%) and blastocyst rates (46.1% and 38.7%). However, the GNT-MP group had lower cleavage (63.4%) and blastocyst rates (18.8%) compared to the two control groups. In the second experiment, the fertilization rate of the control group (76.1%) was higher than the fertilized GNT-MP with 1x106sptz/ml (46.9%) and GNT-MP fertilized with 0.5x106sptz / ml (46%), which did not differ from each other. The polyspermic rate was similar between the control group IVP (18.5%) and the GNT-MP groups either fertilized with 1x106sptz / ml (17.2%) or with 0.5x106sptz / ml (17.5%). It can be concluded that the structures reconstructed by the GNT-MP technique are capable of developing into embryo, and they can be fertilized without increasing the polyspermic rate. Therefore, it is a possible tool for the use of cryopreserved bovine oocytes.

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Regulation of phospholipase C activity reduces premature capacitation of cryopreserved ovine semen

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The objective of the present study was to control the early capacity of cryopreserved ovine spermatozoa by the regulation of phospholipase C (PLC) activity. In Test I, spermatozoa in natura were maintained at 37°C for 4 h in Botubov ® diluent (fraction I) supplemented with 0 (control), 10, 20 or 30 μM of U73122 (Sigma), a PLC inhibitor, in the presence or absence of Glycerol. Parameters of sperm kinetics were evaluated during the incubation period in order to select the doses of the inhibitor to be tested in the cryoprotectant solution (Test II). The cryopreserved ejaculates in Test II were diluted in Botubov (single fraction), plus 0 (control), 10 and 20 μM of U73122. The thawed samples were evaluated for sperm kinetics by the Computer Assisted Sperm Analysis (CASA) system and for membrane integrity by the association of fluorescent propidium iodide, PSA-FITC and JC-1 probes. In addition, evaluations of sperm capacitation and acrosomal reaction were carried out using the fluorescent probe chlortetracycline hydrochloride (CTC) before and after cryopreserved semen samples were submitted to induction of in vitro capacitation in TALP sp medium supplemented with heparin for 4 h Incubation at 37 ° C. All the results were submitted to analysis of variance and Tukey's test, at a 5% probability level. Test I revealed a dose-dependent and time-dependent effect of the PLC inhibitor on the in natura sperm of sheep. In addition, the presence of glycerol enhanced the toxic effect of U73122. In test II, U73122 did not affect any of the kinetic parameters evaluated by the CASA system, nor did the number of spermatozoa with intact or mitochondrial plasma membranes, intact or damaged. However, the evaluations performed immediately after thawing showed that the use of 10 or 20 μM of the inhibitor reduced the percentage of spermatozoa capacitated and with acrosome reacted, in relation to the control group ($p < 0.05$). After induction of the in vitro capacitation, there was a reduction ($p < 0.05$) in the number of unprocessed spermatozoa in all treatments suggesting a reversible effect of U73122 on the process of sperm capacitation and acrosomal reaction. It is concluded that the regulation of PLC activity by the use of U73122, was shown to be efficient in reducing premature capacitation of ovine spermatozoa. In vivo tests should be performed to certify the effect of treatment on the pregnancy rate.

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L-carnitine supplementation during vitrification did not improve survival and quality rates, but altered CrAT and PRDX1 expression in *in vivo*-produced ovine embryos

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Embryo cryodamage is observed mainly at metabolic and molecular aspects and it impairs post warming quality and survival rates. This study aimed to evaluate the effect of L-carnitine (LC) supplementation during either vitrification or post warming solutions on the 6-7th day of in vivo-produced ovine embryos. LC (3.72 mM) was added to vitrification (Experiment 1; C1: control; LC1: supplemented embryos) or warming solutions (Experiment 2; C2; LC2). In vitro culture (IVC) of warmed embryos was performed for 72 h at 38,5 °C, 5% CO2 and 5% O2 to evaluate survival rates in both Experiments. In Experiment 1, reactive oxygen species (ROS) levels were measured by CellROX Green staining, total cell number (TCN) by Hoechst 33342, number of apoptotic cells by caspase-3 immunofluorescence staining protocol, apoptotic index evaluation in both groups. Gene expression analysis of carnitine palmitoyltransferase 1 and 2 (CPT1 and CPT2), carnitine O-acyltransferase (CrAT) and peroxiredoxin 1 (PRDX1), were performed by RT-qPCR (ACTB as endogenous control) in Experiments 1 and 2 and results were compared to fresh embryos (FE). Averages of survival rates were compared by the Chi-Square test. Means of TCN, apoptotic cells, apoptotic index and fluorescence intensity were compared by Student's t-test, at 5% significance level. Survival rates were similar between groups ($p > 0.05$) in Experiments 1 (68.7%, C1 vs 81.8%, LC1) and 2 (48.5%, C2 vs 64.7%, LC2). In Experiment 1, ROS levels at 24 h of IVC (85.83 ± 68.37 x 1010, C1 vs 89.04 ± 84.48 x 1010, LC1), total cell number at 24 h (89 ± 22, C1 vs 82.2 ± 28, LC1) and 72 h (86 ± 19.9, C1 vs 68.5 ± 25.26, LC1), apoptotic cells (3.75 ± 1.48, C1 vs 4.50 ± 4.72, LC1) and apoptotic index (4.37 ± 1.45, C1 vs 5.23 ± 4.72, LC1) at 72 h of IVC did not differ ($p > 0.05$) between C1 and LC1. Gene expression analysis showed no differences in CPT1 and CPT2 mRNA relative abundance in embryos of both experiments compared to FE, however, CrAT was downregulated ($p < 0.05$) in C1 and PRDX1 was downregulated ($p < 0.05$) in both C1 and LC1, compared to FE. Moreover, CrAT and PRDX1 were upregulated ($p < 0.05$) in C2 and CrAT was downregulated ($p < 0.05$) in LC2, in relation to FE. In conclusion, although the short-term LC supplementation at 3.72 mM during cryopreservation did not improve post-warming survival and morphological parameters of the evaluated embryos, it was able to modulate expression of genes related to energy homeostasis (CrAT) and oxidative stress (PRDX1), proving to be beneficial, in both forms of supplementation, to in vivo-produced ovine embryos.

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Survival rates of cryopreserved murine blastocysts exposed to heat stress at 8-cells stage

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Sublethal stress has been reported as inducer of gametes and embryos response, providing cell protection to a subsequent stress. Experiments showed that different treatments using pH modifications, heat and cold shock, osmotic challenge, high environmental pressure, and nutrients starvation lead embryos and gametes to produce different proteins than normally sintethyzed in homeostatic conditions in order to keep favorable conditions facing a next stresfull situation. Within this observation, many researchers started to experiment sublethal stress as protector treatment for cryopreservation. The aim of this experiment was to investigate the use of heat stress of environmental temperature as 8-cells embryos stressor to improve cryopreservation rates at blastocyst stage. Embryos at 8-cells stage were recovered from six weeks old superovulated Mus musculus domesticus females at day 3 pregnancy. Two hundred and twelve embryos were randomly segregated in control (C) and experimental (B) groups. Eight-cells embryos from B group were maintained during 4 hours at 21ºC while control embryos were immediately after recovery in vitro culured in mKSOM media + 0.4% BSA at 37ºC under atmosphere of 5% CO_2, 5% O_2, 90% N_2 and satured humidity. After been exposed to environmental temperature, group B embryos were transferred to the same in vitro conditions as the control group embryos for 48h. Then, embryos from both experimental groups that reached blastocyst stage were cryopreserved in 0.25 mL straws using a classical frozen curve: first, blastocysts were exposed to 1.6 mol of ethylene glicol + mPBS + 0.4% BSA, then cooled at 2 ºC/min to reach seeding (-6 ºC) temperature and then they were cooled at -3 ºC/min until reach - 35 ºC, when they were transferred to liquid azote. Embryo development and expansion rates were compared using Chi-square test ($P < 0.05$) 24h after thawing. Eight-cell embryo developmental rates to blastocyst stage showed no difference among control (95.0% - 92/97) and experimental (95.4% - 110/115) group. Cryopreserved blastocyst expansion rate of stressed embryos was significantly higher (84.5% - 93/110) than cryopreserved control embryos (72.8% - 67/92; $P < 0.05$). We concluded that a simple stress condition like maintaining embryos at environmental temperature (21ºC) can induce a heat stress response that could be usefull to enhance embryo survival after cryopreservation.

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Use of Doppler ultrasonography in embryo transfer programs in the equine species

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In the equine industry, the increase of reproductive efficiency is necessary to reach better results, in addition to increased rate of genetic improvement of livestock. The demand for new biotechnology of assisted reproductive techniques has increased substantially. Ovarian and uterine evaluation of mares by Doppler mode ultrasound helps in the selection of animals that will participate in embryo transfer programs, correlating luteal vascularity with serum levels of progesterone. The aim with this study was to observe results of the assessment of recipients using Doppler mode ultrasound, rectal palpation, cervical evaluation and ultrasound B Mode, in order to compare pregnancy rates between recipients selected by using B Mode ultrasound and using Doppler Mode ultrasound. In this study, follicular dynamics was controlled by rectal palpation and ultrasound examination every day. Two groups of recipients was divided where the first group of recipients (n=14) mares were evaluated by rectal palpation, cervical assessment and B mode ultrasound, while in the second group of recipients (n=15), same parameters were used (rectal palpation, cervical assessment and B mode Ultrasound) and a Doppler Mode Ultrasound examination was added. The evaluations were performed during the selections of recipients at the time of embryo implantation, collected from donor mares at D8 post-ovulation. For statistical analysis calculation Fisher exact test was used. There were 42 embryo collections with a recovery rate of 29 embryos (69%), resulting in 20 pregnancies (68.9%). From these 29 embryos implanted, 14 recipients were evaluated by B Mode Ultrasound where 7 (50%) resulted in positive pregnancy and 15 recipients were evaluated by Doppler Mode Ultrasound and of these, 13 (86.6%) resulted in pregnancy. Despite the low number (n) of recipients evaluated, it was possible to observe a pregnancy rate statistically superior in those animals assessed with Doppler Mode ultrasound compared to the recipients assessed only with B mode ultrasound. In conclusion, the results of this study demonstrated that Doppler Mode Ultrasound presents a large potential for its use and can serve as an important tool in order to optimize selection of recipients that will receive embryos in commercial embryo transfer programs.

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Vitrification of bovine ovarian fragments associate with resveratrol

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In the attempt to minimize morphological damage and the production of metabolites caused by reactive oxygen species from the cryopreservation process, studies have suggested the addition of antioxidants such as catalase, α-tocopherol, trehalose, and resveratrol in vitrification/heating solutions. Resveratrol regulates the expression of SIRT-1 and mitochondrial activity. The aim of this study was to analyze the effect of resveratrol on preantral follicles morphology and, the tissue viability of ovarian fragments from bovine fetuses after vitrification/warning procedures. The ovaries were fragmented and distributed to the control, vitrified and vitrified with resveratrol groups. Preantral follicles were quantified and classified according to the developmental stage. The proportion of normal follicles differed between treatments ($P < 0.05$) and the group of follicles vitrified with resveratrol was superior to the vitrified without the addition of the antioxidant. In resveratrol group, the class of primordial follicles had a higher proportion ($P < 0.05$) of viable follicles. In contrast, the secondary follicle class presented the lowest proportion of normal follicles in both treatments. In addition, a negative association ($P < 0.05$) was observed between the proportion of viable follicles and the stage of follicular development. The probability of finding viable follicles was higher in the group of vitrified follicles in the presence of resveratrol. Moreover, primordial follicles of the resveratrol group had 2.5 times more likely to be viable after vitrification. The chance of observing normal follicles was greater ($P < 0.05$) in the early stages of follicular development. The diameter of the transitional follicles and their respective oocytes were lower when submitted to the vitrification process with resveratrol ($P < 0.05$). In the primary follicle class, follicular and oocyte diameters were similar among the studied groups. The tissue viability performed with confocal microscopy technique evaluated the levels of fluorescence related to reactive oxygen species levels and degeneration levels emitted by diihidrochlorfluorescein and propidium iodide. The fluorescence levels indicating cell degeneration in the group of vitrified fragments with the addition of resveratrol were similar ($P > 0.05$) to the control group. The reactive oxygen species were similar between the vitrified and vitrified groups with the addition of resveratrol. Also, the control group presented a higher level of reactive oxygen species ($P < 0.05$) compared to the other groups. The fluorescence intensity emitted with the use of both probes decreased ($P < 0.05$) with the increased tissue depth. In linear regression analysis, there was a negative correlation between fluorescence intensity and tissue depth. Furthermore, there was a positive association ($P < 0.01$) between degenerate cell levels and the rates of reactive oxygen species produced in treatments with different depths of ovarian tissue, regardless of treatment and tissue depth. Treatment groups showed similar levels of reactive oxygen species. Moreover, the proportion of fragments with high levels of reactive oxygen species was higher in the control group compared to the vitrification treatments. In conclusion, the ovarian tissue fragments of vitrified with antioxidant resveratrol presented a better follicular morphology and tissue viability.