Animal Reproduction

THEMATIC SECTION: 36th ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

SUPPORT BIOTECHNOLOGIES: CRYOPRESERVATION AND CRYOBIOLOGY, DIAGNOSIS THROUGH IMAGING, MOLECULAR BIOLOGY, AND "OMICS

MiRNA characterization of bovine corpus lutem after the administration of different doses of PGF2α on metestrus and diestrus

Lindsay Unno Gimenes[1,2], Marina Anchieta Trevisoli[3], Gabriel Artur Marciano do Nascimento[4], Vanessa Garcia Rizzi[4], Rafael Rodrigues Corrêa[4], Natália Marins Bastos[5], Juliano Coelho da Silveira[6]

[1]Universidade Estadual Paulista
[2]Faculdade de Ciências Agrárias e Veterinárias - Universidade Estadual Paulista "Júlio de Mesquita Filho"
[3]Universidade Estadual Paulista
[4]Ourofino Saúde Animal
[5]Universidade de São Paulo
[6]Universidade de São Paulo
e-mail: lindsay.gimenes@unesp.br

MicroRNAs (miRNAs) are short, non-coding RNAs that act regulating pathways by modulating gene expression. The aim of this study was to evaluate the expression of 39 miRNAs in beef cattle CLs after PGF2α administration, using 50% and 100% of the dose recommended by the manufacturer, in the metaestrus (D4) and diestrus (D11) phases, considering the day of ovulation as D0. The miRNAs were previously selected based on their apoptotic and/or angiogenic functions. Twenty-three females with body condition score of ≥2.75 (score 1-5) were used. The animals were randomized into 4 groups (D4-100%=6; D4-50%=6; D11-100%=5; D11-50%=6). Corpora lutea were obtained after 48 hours of PGF2α administrated and conserved in liquid nitrogen. The miRNAs chosen had functions in apoptosis or/and angiogenesis. For analyses of miRNAs expression in all samples we performed qPCR using reverse transcription with miScript HiSpec Buffer. The relative levels were evaluated and data normalized by miR-99b. Statistical analysis was descriptive, with miRNAs considered as present or absent. It was considered present when in the groups of 6 animals, 5 or more samples had the miRNA, and in the group of 5 animals, 4 or more samples. Exclusive miRNAs (present in a group) were used for the analysis of pathways predicted by the miRWalk 3.0 software. On D4, with 100% of dose, only one exclusive miRNA was found (bta-miR-455-3p), therefore was not possible to perform the pathway analysis. With 50% of the dose, 6 miRNAs were found (bta-miR-132, bta-miR-145, bta- miR-182, bta-miR-378, bta-miR-15a, bta-miR-17-5p), the three pathways with the highest number of associated genes were renal cell carcinoma, adherent junction and inositol phosphate metabolism pathways. In diestrus, 16 exclusive miRNAs were found with 100% of the dose (bta-miR-145, bta-miR-183, bta-miR-202, bta-miR-378, bta-miR-10b, bta-miR-125a, bta-miR-126-5p, bta-miR-15a, bta-miR-17-5p, bta-miR-181a, bta-miR-20a, bta-miR-26a, bta- miR-29a, bta-miR-34a, bta-miR-455-3p and bta-miR-92a), and none when 50% of the dose was used. The three main pathways of D11-100% were metabolic, cancer and MAPK signaling pathways. The bta-miR-455-3p, exclusive to D4-100%, is related to apoptosis in cancer cells, and may be associated with an initiation of luteolysis. In the D4-50% group, activation of the renal cell carcinoma and adherent junction pathway is supposed due to the cell multiplication phase and structural formation of the CL, and inactivation of the Inositol phosphate pathway, since it is a pathway present in luteolysis. In D11-100%, the metabolic pathway can be associated with a possible decline in P4 serum, and the activation of cancer and MAPK signaling pathways, possibly participating in the apoptosis process caused by luteolysis.

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Post-warming culture with beta-mercaptoethanol does not alter embryo survival but improves the quality of in IVP bovine embryos post vitrification

Viviane Luzia da Silva Feuchard[1,2], Leticia Zoccolaro Oliveira[1], Naiara Zoccal Saraiva[2], Clara Slade Oliveira[2]

[1]Universidade Federal de Minas Gerais
[2]Embrapa
e-mail: viviane.lfeuchard22@gmail.com

The aim of the present study was to investigate the relationship between vitrification and excessive generation of reactive oxygen species (ROS) and the effects of oxidative stress attenuation on the survival and quality of bovine in vitro produced (IVP) embryos. To test the hypothesis that oxidative stress was related to lower post-vitrification embryo survival, the antioxidant beta-mercaptoethanol was used in the post-warming culture and then the survival and quality of embryos were assessed. In experiment 1, we evaluated the levels of ROS in grade I blastocysts (at day 7 after IVF) from fresh (F) and post-warming vitrified (V) embryos. In experiment 2, F and V embryos were submitted to 48 hours of culture (SOF) in the presence (F+, V+) or absence (F-, V-) of beta-mercaptoethanol (100 µM) (Gibco BRL, Grand Island, NY) for posterior assessment of re-expansion, hatching, number of cells and oxidative index. All IVP stages (as well as post-warming culture) were carried out at 38.5ºC in 5% CO2 in high humidified air. Re-expansion was evaluated at 0, 2 and 4 hours after warming. In both, experiments 1 (2h after warming) and 2 (48 h after warming), the embryos from each group were stained with CellRox Green (Invitrogen Molecular Probes, USA) and Hoechst 33342 (Sigma-Aldrich, USA). Then, images of each embryo were captured and evaluated using ImageJ software to measure the levels of ROS (average pixels) and number of cells. According to the present results, increased concentration of ROS was detected in vitrified embryos considering the fluorescence levels (F= 57.25±4.17, V= 103.71±8.68, P<0.05; T Test, n= 117, 53-64 per group). No differences were observed regarding the re-expansion rate of vitrified embryos cultured in the presence or absence of beta-mercaptoethanol (0 hrs: V+ = 63.86%, V- = 62.64%; 2hs: V+ = 90.36%, V- = 95.60%; 4 hs: V+ = 92.77%, V- = 98.90%, P>0.05, Fisher's Exact Test, n= 174, 83-91, per group). The hatching rates of vitrified embryos (V+ and V-) were similar to F- embryos but lower than F+ embryos (V+ = 49%, V- = 48%, F+ = 71%, F- = 66%, P<0.05, Exact Test Fisher, n=229, 79-81-34-35, per group, respectively). Still, the total number of cells in V+ embryos was similar to F- and F+ blastocysts (F- = 158.04±14.53, F+ = 163.19±9.21, V- = 117.40±5,50, V+ = 148.04±7.26, P>0.05, Post Tukey Test, n= 214, 23-32-81-78, per group, respectively). The oxidative index (at the end of 48 h of culture) was not different among the experimental groups (F- = 309.51±29.25, F+ = 342.67±28.26, V- = 384.19±26.65, V+ = 375.49±26.99, P>0.05; Post Dunn's Test, n=196, 22-32-67-75, per group, respectively). We conclude that, although it did not affect embryo survival, the attenuation of oxidative stress improved the quality of vitrified embryos.

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Exploring synthetic liposomes as carriers for bioactive molecules in animal reproduction: characterizing vesicles and investigating endocytosis in granulosa cells

Matheus Andrade Chaves[1], Mariani Farias Fiorenza[1], Samantha Cristina de Pinho[1], Flavio Vieira Meirelles[1], Felipe Perecin[2,1], Juliano Coelho da Silveira[3,1]

[1]Faculdade de Zootecnia e Engenharia de Alimentos - Universidade de São Paulo
[2]Universidade de São Paulo
[3]Universidade de São Paulo
e-mail: matheus.chaves@usp.br

Liposomes (LPs) are spherical vesicles composed of a lipid bilayer membrane that can encapsulate various types of molecules, including drugs, proteins, and genetic material. In the specific case of in vitro production (IVP) of embryos, these carriers can be used to deliver DNA or RNA constructs encoding for genes of interest, such as growth factors or transcription factors, into the developing embryo. This can potentially improve the development and quality of the resulting embryos, as well as allow the production of genetically modified embryos for research purposes. In this study, LPs were produced using phosphatidylcholine and phospholipids labeled with a rhodamine fluorophore (Avanti Polar Lipids, USA). The LPs were produced through ultrasonication followed by extrusion using 100 nm polycarbonate membranes. Size, distribution, and zeta potential of vesicles were determined using dynamic light scattering (DLS). Concentration was assessed by nanoparticle tracking analysis (Nanosight 3000, Malvern P., UK). All parameters were evaluated using Tukey's test at $p < 0.05$ to determine the significance of differences for three technical replicates used to produce LPs. The labeling efficiency of vesicles with the fluorophore was assessed through flow cytometry (Cytoflex, Beckman C., USA). To assess the potential and future applications of these LPs in transfecting bioactive molecules, they were added to granulosa cells (GCs) *in vitro* culture to verify endocytosis. The CGs were obtained from 3 to 6 mm follicles of bovine ovaries collected from a slaughterhouse through dissection and cultivated in 6-well cell culture plates using FSH-supplemented DMEM-12 medium. Samples obtained by adding LPs to CGs included the following groups: C1 (pure culture), C2 (culture with no-labeled LPs) and T (culture with labeled LPs). The parameters evaluated included cell concentration in culture, concentration of LPs added to the culture, concentration of labeled lipids in the LPs formulation, and duration of incubation. The evaluation of endocytosis was conducted through image analysis, using a fluorescence microscope (THUNDER 3D Imager Systems, Leica M., Germany). The analysis was performed with three biological replicates. The preliminary results indicated that LPs have a diameter of approximately 150 nm and a homogeneous distribution. Also, zeta potential was around -40 mV, indicating stability in dispersion. Optimal images were obtained when concentrations of around 109 liposomes/mL were added to cultures containing 105 granulosa cells per well. The images demonstrated that liposomes were endocytosed within 6h and remained localized in the perinuclear region of the cells. In the future, confirmation of endocytosis will enable the incorporation of bioactive molecules of interest for IVP into these promising LPs. It will also reveal whether such a phenomenon continues to occur when using modified LPs.

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Cryotolerance and lipid droplet accumulation in bovine embryos cultured *in vitro* in the presence of fetal calf serum or yeast extract: preliminary results

Luany Alves Galvão Martinhão[1], João Gabriel Viana de Grázia[2], Leticia Prates Martins[1], Otávio Augusto Costa de Faria[1], Dhonata Nunes Ribas[3], Ismael do Nascimento Garcia[3], João Henrique Moreira Viana[4]

[1]Universidade de Brasília
[2]Apoyar Biotech
[3]Universidade do Estado de Mato Grosso
[4]Embrapa Recursos Genéticos e Biotecnologia
e-mail: luany.galvao@gmail.com

The use of bovine fetal calf serum (FCS) as a medium supplement is associated with greater embryo rates, but also with lower cryotolerance and large offspring syndrome. Different supplements have been tested as potential alternatives for FCS. Results of the doses- response our group reveals that the yeast extract (YE) can be used during the *in vitro* culture (IVC) at 1% (0.1mg/mL). The aim of the study was to evaluate lipid droplet accumulation and embryo hatching after cryopreservation when FCS has replaced by YE 1%. Bovine *cumulus* oocyte complexes (COC) were recovered from slaughterhouse ovaries (Nelore breed, *Bos indicus*). *In vitro* maturation and fertilization were performed for 22h and 20h, respectively, following the standard protocols of the commercial North Embryo laboratory - Alta Floresta, Brazil. The presumptive zygotes were randomly distributed into five groups, all submitted to IVC in SOF media added with BSA (3mg/mL) from days 1 to 9 and supplemented with: G1, 3% FCS (n=282); G2, no supplement (n=285); G3, YE 1% (n=271) during all IVC; G4, YE 1% only up to day 3 of IVC (n=267) and G5, YE 1% up to day 3 and 3% FCS from days 3 to 9 of IVC (n=272). Lipid droplet accumulation was evaluated in a subset of day-7 blastocysts (n=92 in four replicates). Hatching rate at day 9 of IVC was evaluated on fresh blastocysts (n=506 in five replicates) and on vitrified-warmed embryos (n=360 in nine replicates). Data were analyzed using the Glimmix procedure (SAS software), considering the effects of treatment, replicate, and their interaction. There was no replicate effect (P>0.05) for any of the endpoints analyzed. Cultured embryos without supplementation (G2) presented lesser lipid droplets than those supplemented with FCS, YE 1% or YE 1% + FCS (G2: 7.6%±1.1a vs. G1: 14.8%±1.3bc; G3: 15.5%±1.5bc; G4: 17.2%±2.3c, P=0.0001), excepting the YE 1% up to day 3 of IVC (G5: 9.6%±1.2ab), being similar; embryos were stained with Bodipy and evaluated under a laser-scanning confocal microscope. Greater hatching rates after blastocyst vitrification-warming were obtained when FCS was used throughout CVI (G1: 78.9%±0.6a; vs. G2: 39.4%±0.7b G3: 46.6%±0.7b G4: 51.3%±0.8b and G5: 32.8%±0.8b, P=0.0001). For fresh blastocysts, the hatching rate of the group supplemented with FCS (G1: 74.0%±0.8a) was similar (P>0.05) to those supplemented with YE 1% throughout IVC (G3: 61.8%±0.7ab) or with YE up to day 3 followed (G5: 70.0%±0.8a) or not (G4: 76.0%±0.8a) by FCS from days 3 to 9, but greater (P=0.0073) than in groups without supplementation (G2: 48.2%±0.5b). In summary, the potential effects of YE during IVC depend on the fate of the embryos (fresh or vitrified). In fresh blastocysts, YE didn't impair hatching. However, when blastocysts are not supplemented or to with YE, it causes a reduction in hatching after vitrification-warming compared to with FCS, suggesting that cryotolerance is affected by factors other than lipid accumulation, for example, nutrient insufficient.

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The use of follicular fluid in the vitrification of bovine oocytes increases the expression of *HSP70*

Leonardo Henrique da Silva Nogueira[1], Marcela Oliveira das Mercês[2], Eduardo Baia de Souza[3], Bruno Porpino Homobono[1], Ana Paloma Lobo Cardoso[3], Matheus Hadigo Pires Costa[1], Marcela Silva Cordeiro[4], Nathalia Nogueira da Costa de Almeida[5], Simone do Socorro Damasceno Santos[1], Priscila di Paula Bessa Santana[2]

[1]Universidade Federal do Pará
[2]Universidade Federal Rural da Amazônia
[3]Universidade Federal do Pará
[4]Instituto Federal de Educação, Ciência e Tecnologia do Pará
[5]Universidade Federal do Pará
e-mail: leonardo.nogueira@icb.ufpa.br

Bovine follicular fluid (BFF) has replaced fetal bovine serum (FBS) in oocyte maturation media due to its composition being rich in proteins, metabolites and growth factors, in addition to its low cost. However, there are still no studies on this substitution in vitrification protocols. This study aimed to investigate the effects of BFF and FBS on vitrification of bovine cumulus oophorus complexes (COCs), evaluating their impact on post- warming survival through their effect on heat shock protein 70 (HSP70) gene expression. Grade 1 COCs, obtained by puncturing ovaries from a local slaughterhouse, were matured *in vitro* (TCM 199 supplemented with 25 mM sodium bicarbonate, 10% FBS, pyruvate, gentamicin, FSH and LH, covered with mineral oil and kept at 38.5° C, 5% CO2, for 22 hours). After maturation they were randomly divided (30 per group) into three experimental groups: FBS group - vitrified in TCM 199 supplemented with 20% FBS and cryoprotectants: 10 or 20% (SV1 and SV2) ethylene glycol (EG) and dimethylsulfoxide (DMSO) and 0.5 M sucrose; BFF group - vitrified in TCM 199 supplemented with 20% BFF and cryoprotectants; and non-vitrified group, which were immediately denuded and stored in RNAlater (Applied Biosystems©). Furthermore, two equilibration times were used in the first vitrification step (5 or 3 minutes), resulting in additional experimental groups (FBS3' and BFF3') and (FBS5' and BFF5'). Gene expression of COCs was evaluated by real-time PCR using the StepOne plus® system (Applied Biosystems©). All statistical analyzes were performed using Sigma plot 14.5 software ($p<0.05$) (values shown represent the mean of $2^{-\Delta\Delta CT}$). Regarding HSP70 expression, the experiment did not show significant differences in oocytes vitrified with FBS (5,145) or BFF (3,997) ($p>0.05$), but both groups differed from the control group (1,069) ($p<0, 05$). This study showed significant differences in the groups exposed for 3 minutes (FBS and BFF 3') in relation to the groups exposed for 5 minutes regarding the expression of the HSP70 gene, which was higher in the groups of 3 minutes ($p<0.05$). This could be a cellular response to heat stress, as well as an attempt to combat apoptosis, as heat stress damages the cell membrane. Likewise, the decrease in HSP70 expression in oocytes exposed to the 5-minute groups may indicate, in addition to the low survival rates of oocytes after vitrification, that the cells were already in the process of apoptosis. This same result was also observed in cumulus cells from immature oocytes. Vitrification with BFF is an unprecedented method that needs to be improved to make it more viable and reduce the rate of cell degeneration, modifying the concentration of cryoprotectants and the time of exposure. Thus, studies are needed to investigate the structural changes in cells as well as the biochemical influences of BFF to prove its usefulness in vitrification protocols.

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Oxidative metabolism alters histone lactylation in pre-implantation bovine embryos

João Vitor Alcantara da Silva[1], Jessica Ispada[2], Aldcejam Martins da Fonseca Junior[2], Patricia Kubo Fontes[3], Marcella Pecora Milazzotto[2]

[1]Universidade Federal do ABC
[2]Universidade Federal do ABC
[3]Universidade Federal do ABC
e-mail: vitoralcantaraj@gmail.com

The Warburg effect, characterized by the production of lactate via aerobic glycolysis, is an important metabolic pathway during the pre-implantation embryonic development, especially from the morula stage. Recently, a new role for lactate was described, its use for a previously unknown histone mark, the histone lysine lactylation (KLa). Our group early demonstrated the presence of KLa in the morula stage of bovine embryos. However, it remains unknown whether KLa is also present and regulated by the oxidative metabolism in the blastocyst. The objectives of this study were: 1) to verify the presence of lysine histone lactylation (Kla) in bovine blastocyst (day 7) and 2) to evaluate the global levels of Kla in bovine blastocysts cultured in two different oxygen tensions (O2): lower oxygen tension [5%O2] or atmospheric O2 concentration [20%O2]. To this end, embryos were in vitro produced by standard protocols. After fertilization, the presumptive zygotes were randomly divided in two groups 5%O2 or 20%O2 and cultured in Embryonic Culture System 50 medium (ECS50),) supplemented with 4 mg mL−1 BSA until day 7. The mitochondrial membrane potential (MMP) was determined by the Mitotracker TM Red CMXRos dye (ThermoFisher) - n = 30 blastocyst/for each group - the global levels of lactylation were determined by immunostaining using the Polyclonal antibody pan histone lactylation (PTM-1401) - n = 30 blastocyst/for each group. The images were acquired using a fluorescence microscope and analyzed by ImageJ software. Data were analyzed by Student"s t-test considering P 0.05. Higher levels of MMP and histone lysine lactylation were found in 20%O2 when compared to 5%O2 (MMP - 0.68±0.3 and 0.37±0.2, respectively, p 0.001; KLa - 2.4±1.5 and 1.4±1.2 , respectively, p=0.010). This suggests that, in the presence of high levels of O2 (20%) there is an expected increase of oxidative metabolic pathways, such as the mitochondrial oxidative phosphorylation, but also the aerobic glycolysis, which probably increases the lactate availability in the blastocyst. Deeper analysis showed that both groups presented higher levels of Kla in the Inner Cell Mass (ICM) than the trophectoderm (5%O2 - ICM: 0.9±0.6; trophectoderm: 0.7±0.6, respectively, p=0.0230; 20%O2: ICM: 1.3±0.8; trophectoderm: 1.1±0.7, respectively, p=0.0064). In conclusion, as far as we know, this work describes for the first time the lysine histone lactylation in bovine blastocysts and suggests that different availability of O2 during in vitro culture induces changes in the balance between glycolysis and mitochondrial metabolism, consequently shaping the epigenetic mechanisms that act by regulating the embryonic transcriptional machinery.

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Effect of resveratrol exposure before or after vitrification of immature COCs on gene expression and nuclear maturation in feline species

Thais de Almeida de Oliveira[1], Gabriela Ramos Leal[2], Mariana Pedrosa de Paula Guimarães[3], Lucas Francisco Leodido Correia[1], Viviane Lopes Brair[1], Ana Lucia Rosa e Silva Maia[1], Ribrio Ivan Tavares Pereira Batista[1], Joanna Maria Gonçalves de Souza Fabjan[1]

[1]Universidade Federal Fluminense, [2]Universidade Federal Fluminense, [3]Universidade Federal Fluminense
e-mail: almeidathais@id.uff.br

One of the biggest challenges of oocyte vitrification is reducing the cryo injuries, caused by excessive use of cryoprotectants, osmotic and oxidative stress. This study assessed the effect of resveratrol (RESV), as an antioxidant, either before or after the vitrification of feline COCs on nuclear maturation and gene expression. For this, COCs were recovered from feline ovaries obtained in elective surgeries. COCs presenting homogeneous cytoplasm and surrounded by, at least, one layer of cumulus cells, were selected and vitrified in eight replicates, according to the following groups: Vitrified without any exposure to RESV (VIT, n=49), exposed for 90 min to RESV before vitrification (RESV-VIT, n=52), or exposed for 90 min to RESV after vitrification (VIT-RESV, n=52). In addition, two experimental groups containing fresh oocytes were also assessed, exposed (CONT-RESV, n=69) or not (CONT, n=62) to RESV. Exposure was carried out in TCM 199 supplemented with 1 mmol/L of pyruvate, 4 mg/mL of BSA, 100 µL/mL of penicillin-streptomycin, and 1 µM of RESV, at 38.5°C, in a humidified atmosphere of 5% CO2. After warming, three pools of five immature oocytes from each group were frozen to further assess the expression of BAX, HSP70, GDF9, ZAR-1, BMP15, SIRT1, and SIRT3 genes (normalized by YWHAZ, ACTB genes), and the remaining COCs were submitted to IVM (TCM199 supplemented with 0.02 UI/mL FSH/LH, 100 µM cysteamine, 0.5 mg/mL L-carnitine, 2.2 g/L sodium bicarbonate, 3 mg/mL BSA, 0.25 mg/mL sodium pyruvate, 0.15 mg/mL L-glutamine, 0.6 mg/mL sodium lactate and 0.55 mg/mL gentamicin), for 28 h, at 38.5 °C, in an atmosphere with 5% low O2 tension in six replicates. Relative quantification was performed by the comparative Ct method ($2^{-\Delta\Delta Ct}$) for gene expression and Fisher's exact test for IVM. In the nuclear maturation assessment, CONT-RESV had a higher ($P<0.05$) MII rate (51.9%) than CONT (31.9%), VIT (20.6%), RESV-VIT (16.2%), and VIT-RESV (24.3%), which were all similar ($P>0.05$) to each other. The ZAR-1 gene showed a higher ($P<0.05$) expression in RESV-VIT compared to VIT. Both vitrified groups exposed to RESV showed higher expression of GDF9 compared to the not- exposed group (VIT). Both BPM15 and PRDX1 transcripts were upregulated in all RESV- exposed groups (RESV-VIT, VIT-RESV, and CONT-RESV) compared to the not-exposed ones (VIT and CONT). SIRT1 was upregulated in all RESV-exposed groups compared to CONT, while RESV-VIT showed higher expression compared to VIT. SIRT3 was upregulated in CONT-RESV compared to CONT and VIT. HSP70 was upregulated in all groups compared to CONT. BAX was upregulated in the CONT-RESV compared to CONT, VIT, and RESV-VIT groups. In conclusion, although not affecting nuclear maturation, RESV seems to improve the quality of IVM-oocytes previously subjected to vitrification, based on the expression of genes related to developmental competence and metabolism.

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Antifreeze protein type I improves vitrification outcomes in cat cumulus-oocyte complexes

Mariana Pedrosa de Paula Guimarães[1], Lucia Prellwitz Murad[1], Thais de Almeida de Oliveira[1], Gabriela Ramos Leal[1], Lucas Francisco Leodido Correia[1], Nathalia Oliveira Barbosa[1], Ribrio Ivan Tavares Pereira Batista[1], Joanna Maria Gonçalves de Souza Fabjan[1]

[1]Universidade Federal Fluminense
e-mail: marianappguimaraes@gmail.com

The domestic cat is an important experimental model to apply reproductive biotechnologies aiming at the conservation of endangered species of felids. The use of antifreeze protein I (AFP I) in the cryopreservation of female gametes is a tool to minimize adverse effects caused by the cryopreservation process. This study aimed to evaluate two different concentrations of AFP I added in the vitrification solution and their effects on nuclear maturation and gene expression profile of vitrified cat oocytes. To reach that, COCs were recovered from feline ovaries obtained in elective surgeries and, after selection (only Grade 1 and 2), were allocated into three groups (GO, G0.5, and G1) and vitrified. Vitrification was performed by exposing the COCs in equilibrium solution [TCM-199 Hepes-buffered plus 20% fetal bovine serum (Base medium, BM), 7.5% ethylene glycol (EG) and 7.5% DMSO dimethyl sulfoxide (DMSO)] with 0 µM (G0), 0.15 µM, (G0.5) or 3.0 µM (G1) of AFP I for 15 m, and then, transferred to vitrification solution (BM with 15% EG and 15% DMSO) containing the same concentration of AFP I (depending on the group) for up to 90 s. After that, COCs were disposed of on Cryotop® devices in groups of two to six and immersed in liquid nitrogen. For warming, COCs were placed in a solution containing 1 mol/L of sucrose dissolved in BM at 37 ºC for 1 m and after that, in a solution with 0.5 mol/L of sucrose in BM for 3 m. Then, the structures remained for 5 m in a BM solution without sucrose. After warming, most of the oocytes from the three vitrified groups and from a new fresh group (GF) were submitted to IVM (TCM-199 HEPES, 3 mg/mL of BSA, 10 µL/mL of pyruvate, 5 µL/mL of glutamine, 10 µL/mL of sodium lactate, 10 µL/mL of penicillin-streptomycin, 5 µL/mL of cysteamine, 10 µL/mL of FSH, 10 µL/mL of L-carnitine) in four replicates at 38.5 °C, in 5% O2, 5% CO2, and 90% N2. After IVM, for nuclear maturation assessment, COCs (n=290) were denuded using hyaluronidase, fixed in 4% PFA for 40 m, and stained with HOECHST 33342 for evaluation. Also, three pools of five oocytes from each group were selected for the expression of HSP70, PRDX1, GDF9, ZAR1, BMP15, SIRT1, and SIRT3 genes (normalized by ACTB and YWHAZ genes). Regarding nuclear maturation assessment, there was no difference ($P>0.05$) among the vitrified groups supplemented with AFPI [G0.5 (60.6%) x G1 (43.7%)], however, both groups presented a higher ($P<0.05$) maturation (MII) rate compared to G0 (20.0%) and a lower rate compared to GF (92.5%). In comparison with GF, both G0 and G0.5 presented a downregulation ($P<0.05$) of HSP70, PRDX1, GDF9, and ZAR1 genes, while G1 was similar ($P>0.05$) to GF. Conversely, G1 showed a downregulation ($P<0.05$) of HSP70 and ZAR1; and an upregulation ($P<0.05$) of SIRT3 compared to GF. It was concluded that the supplementation of AFP I in the vitrification media improves nuclear maturation rates and molecular aspects of cat oocytes.

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Qualitative metabolomic profile of fluid from follicles of different sizes in ewes

Irving Mitchell Laines Arcce[1], Lorena Mara Alexandre e Silva[2], Kirley Marques Canuto[2], Elenilton de Godoy Alves Filho[2], Maiana Silva Chaves[1], Natanael Aguiar Braga Negreiros[1], Luciana Magalhães Melo[3], Maurício Fraga van Tilburg[1], Vicente José de Figueiredo Freitas[4]

[1]Universidade Estadual do Ceará
[2]Embrapa
[3]Centro Universitário Fametro
[4]Universidade Estadual do Ceará
e-mail: irving.laines@aluno.uece.br

Follicular fluid (FF) is formed from the transudation of theca and granulosa cells in the growing follicular antrum. Its main function is to provide an optimal intrafollicular microenvironment to modulate oocyte maturation. The aim of this study was to determine the metabolomic profile of FF of ewes collected from follicles of different sizes. Ovaries (n=36) were collected from 18 adult ewes (2-4 years old) from a local slaughterhouse and transported at 37°C to the laboratory in 0.9% saline with antibiotics. Ovaries were washed three times in transport solution at 37.5 °C and kept in a water bath. Soon after, with the help of a vernier rule, the follicle was determined. Thus, the FF was collected from small (< 3 mm, n=27) and large (≥ 3 mm, n=19) follicles using a 20 G needle attached to a 5 mL syringe. The total volume recovered was 370 µL from small and 800 µL from large follicles. Samples were pooled and centrifuged at 3350 rcf at 4° C for 15 min. Then, the supernatant was recovered and kept in tubes at -80° C until analysis. Then, FF samples (150 µL) from each group were analyzed by high-resolution hydrogen nuclear magnetic resonance (1H NMR) spectroscopy. Samples were prepared by mixing 150 µL of FF and 400 µL of deuterated water solution with 0.2 M Na2PO4/NaH2PO4 and 0.4 mM of TMSP-2,2,3,13-d4 (sodium trimethyl propionate). Data were qualitatively evaluated. Eighteen different metabolites were observed among amino acids, carbohydrates and intermediate metabolites. Different spectral signal intensities were also observed according to follicles for almost identified metabolites. When compared to large follicles, small ones showed, apparently, a greater spectral signal for alanine, choline, choline phosphate, myo-inositol and glycerol. In conclusion, the present study provides novel information on the FF metabolic profile in ewes. The observed metabolites have different roles in the oocyte development and maturation. However, quantitative determination is essential for more accurate conclusions.

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Impact of lipid modulation on bovine oocyte and embryo cryotolerance: a systematic review

Erlandia Márcia Vasconcelos[1], Rachel Ferreira Braga[1], Gabriela Ramos Leal[1], Thaís Gomes de Oliveira[1], Felipe Zandonadi Brandão[1], Joanna Maria Gonçalves de Souza Fabjan

[1]Universidade Federal Fluminense
e-mail: erlandiamvasconcelos@gmail.com

Attempts to improve the cryopreservation technique of oocytes and embryos have been carried out to optimize the IVP and one of the strategies is the lipid modulation of the oocytes and embryos. The impact of reducing lipid content to improve cryopreservation has been widely studied. However, there is no consensus on the real effect of lipid accumulation during IVM and IVC, regarding cryotolerance. Thus, this study systematically analyzed in original articles the impact of reducing the lipid content in IVM and/or IVC on the cryotolerance of bovine oocytes and/or embryos. Scopus, PubMed, and Web of Science databases were searched; date range filters were not applied. Articles using lipid modulators in bovine oocytes (at IVM) or embryos (at IVC), as well as having a control group (no modulation), and that evaluated survival rates after cryopreservation were included. The experiments were classified as "positive", when any concentration of the intervention used led to an increase in survival; "with no effect", if the effect was similar to the control group; and "negative", when presented a reduction in survival rates. When the article tested different concentrations, survival could be positive, with no effect, or negative, according to the concentration of the lipid modulator used. A total of 765 articles were obtained on the searches and 35 were included in this study. Out of those, 63 experiments were extracted, eight performed at IVM and 55 at IVC. A total of 14 substances were tested as potential lipid modulators and the number of experiments carried out with each substance were respectively: L-carnitine (LC; 20), conjugated linoleic acid (CLA; 14), forskolin (FK; 7), albumin linoleic acid (ALC; 6), phenazine ethosulphate (PES; 5), linoleic acid (LA; 2), serum replacer (SR; 2), diacylglycerol acyltransferase-1 (DGAT1; 1), sericin (SE; 1), cyclic guanosine monophosphate (cGMP; 1), niacin (NI; 1) docosahexaenoic acid (DHA; 1), PES+LC (1), CLA+LC (1). In the experiments performed during IVM, only two (2/8) using NI and LC had positive results in survival. One (1/8) had a negative effect, and all others (5/8) had no effect. In experiments assessing the IVP-embryos cryosurvival, 42% (23/55) had a positive result (LA = 1; CLA = 3, LAA = 4; PES = 3, LC = 8, FK = 2, SR = 2), 44% (24/55) had no effect, 5% (3/55) had a negative effect, and 9% (5/55) showed both negative and positive results, depending on the concentration. In conclusion, although modulators do not seem to impair cryosurvival, its improvement was not unanimous, especially during IVM, showing that cryotolerance may not be greatly impacted by lipid modulation. Finding which lipids are reduced can help the understanding of the real relationship between lipid content and the cryosurvival of bovine oocytes and embryos.

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High hydrostatic pressure of 100 or 300 bar is not capable to increase embryonic development of cryopreserved immature bovine oocytes

José Eduardo Vieira Chaves[1], Laryssa Ketelyn Lima Pimenta[1], Gabriela Martins de Almeida[1], Emanuely Zequim Ubeda[1], Alice Caroline Souza[1], Marcos Coura Carneiro[1], Leonardo de França e Melo[1], Emivaldo de Siqueira Filho[2], Margot Alves Nunes Dode[3], José Felipe Warmling Sprícigo[1]

[1]Universidade Federal de Goiás
[2]Embriotec - Reprodução Animal
[3]Embrapa Recursos Genéticos e Biotecnologia
e-mail: josechaves@discente.ufg.br

High Hydrostatic Pressure (HHP) promotes a sublethal stress on cells, affecting molecular pathways and potentially increases cell resistance. The objective of the present study was to evaluate the embryonic development of immature COCs submitted to vitrification after HHP. Grade I and II COCs obtained from slaughterhouse ovaries were used. For HHP treatment, groups of 25-30 COCs were maintained in 0.25ml straws in TCM-199 medium, with 10% FCS, the straws were maintained in the pressure chamber (Cryo-Innovation) at 100 bar (Experiment1) or 300bar (Experiment 2), for 30 minutes. Oocytes from the cryopreserved groups were vitrified and warmed in accordance to Cryotop methodology (Kitazzato). Groups of COCs not submitted to pressure and/or vitrification, remained under a heating plate, in same holding medium at 36º C until the end of all treatments. For each experiment, immature COCs were distributed into five experimental groups: COCs not cryopreserved and not subjected to pressure served as controls (Cont); COCs subjected to pressure but not vitrified (ContHHP); COCs only submitted to vitrification (ContVit); COCs vitrified 60 min after HHP (Vit60) and COCs vitrified 120 min after HHP (Vit120). Oocytes from all groups returned to the incubator until completing 22 hours of IVM, then IVF and IVC. Cleavage at D2 and blastocyst rates at D7 and D8 were evaluated. Data were analyzed using the Chi- square test, with a significance of 5%, the experiments were independent being pressure sections (100 and 300 bar), statistical analysis and experimental groups separated by experiment and not compared with each other (experiment 1 vs experiment 2).In experiment 1 (100 bar), all vitrified groups ContVit (n=118), Vit60 (n=158) and Vit120 (n=156) were similar (P>0.05) in terms of cleavage rate (16.1%; 14.6% and 15.4%) and blastocysts in D7 (4.2%; 1.9% and 1.9%) and D8 (3.4%; 1.9% and 2.6%). Fresh groups, Cont (n=154) and ContHHP (n=109) presented higher (P<0.05) cleavage (75.3% and 67.9%) and blastocyst rates at D7 (31.8% and 19.3%) and D8 (32.5% and 22.9%) compared to vitrified ones. However, on D7 the embryonic development of the ContHHP group was lower (P<0.05) compared to Cont. In experiment 2 (300 bar), all vitrified groups ContVit (n=78), Vit60 (n=93) and Vit120 (n=114) were similar (P>0.05) in terms of cleavage rate (17.9%; 14.0% and 13.2%) and blastocyst at D7 (0%; 0% and 1.8%) and D8 (0%; 0% and 1.8%). Fresh groups Cont (n=85) and ContHHP (n=66) presented higher (P<0.05) cleavage rates (71.8% and 56.1%), blastocyst at D7 (35.3% and 22.7%) and D8 (37.6% and 24.2%) compared to vitrified groups. However, on D2 the cleavage of ContHHP was lower (P<0.05) than in Cont group. In summary, exposing the immature COC to 100 bar or 300 bar is not capable of increasing the resistance of oocytes to vitrification/warming. Furthermore, in both experiments, pressure negatively affected the embryonic development of fresh oocytes at some point of development.

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Multiple mutations can be found in the exon 11 of prolactin receptor gene in crossbred bovine embryos

Diana Rangel de Lemos[1], Jessica Fernanda da Silva e Souza[2], Eliza Diniz de Souza[3], Naiara Zoccal Saraiva[3], Carolina Capobiango Romano Quintão[3], Luiz Gustavo Bruno Siqueira[3], Clara Slade Oliveira[3], Luiz Sérgio de Almeida Camargo[3]

[1]Universidade Federal de Viçosa
[2]Universidade Federal de Juiz de Fora
[3]Embrapa Gado de Leite
e-mail: dianalemosvet@gmail.com

Cattle with the SLICK haplotype have been characterized by a sleek and short hair coat, and one of the primary benefits of the SLICK haplotype is its role in improving thermoregulation in cattle, particularly in hot and humid climates. Causal variant responsible for the slick phenotype in cattle is primarily located in the 11th exon of the prolactin receptor gene, however it should be noted that not all variants found in this region result in the slick phenotype (Porto-Neto et al., Front. Genet., 9:57, 2018). Nevertheless, these single alleles remain crucial for matters of guide design in CRISPR experiments, particularly those aimed at knocking out or modifying the prolactin receptor gene. The identification of these single alleles contributes to a more comprehensive understanding of genetic variation in the region and can assist researchers in designing more precise and effective guide RNAs for their experiments. Consequently, even alleles that do not directly contribute to the slick phenotype possess significant value in advancing our knowledge of the underlying genetic mechanisms implicated in this essential trait. The aim of this study was to assess the genome sequences of in vitro fertilized (IVF) Bos taurus x Bos indicus crossbred cattle embryos, with a particular focus on the PRLR region. Blastocysts were individually collected and subjected to DNA extraction using a two-step incubation method with proteinase K (1,5ug/uL) lysis buffer. Subsequently, PCR amplification was conducted in duplicate and the PCR fragments were submitted to Sanger sequencing. Sequence analysis was performed using Unipro Ugene software (Okonechnikov K., et al. Bioinformatics, 28 (8):1166-7, 2012). A total of 15 samples were analyzed and it was observed that 33.3% (5/15) of the samples exhibited a single mutation (C>T) at position 39099463, resulting in a substitution from a serine to a stop codon that has not been reported before. In addition, a pair of missense mutations were identified in a closely located region, with position 39099322 showing a mutation (G>T) from an arginine to a leucine in 60% of the samples, and position 39099190 exhibiting a mutation (C>T) from a serine to a leucine in all samples. Lastly, a silent mutation was identified at position 39099368, potentially resulting in a substitution of cytosine by thymine in 60% of the samples, which would lead in both cases to the synthesis of a tyrosine. Based on the results obtained from the initial analysis, it can be inferred that this region has an increased potential for genetic variation. Therefore, it is recommended to inspect the target genomic region of crossbred animals and compare with Bos taurus one before designing guide RNAs aiming to introduce indels to promote the slick phenotype. In conclusion, this study's findings provide valuable insights into the genetic variation of the PRLR region in cattle, which may influence the gene editing efficiency.

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Effects of exposure of in vitro produced bovine embryos to moderate hypothermia: preliminary results

Luciano Cruz Pantoja[1], Alysson Jorge Oliveira Sousa[2], Izamara do S. R. Rodrigues[1], Paula Sabrina Arruda Coelho[3], Lidiany C. Aleixo[1], Aryadne L. Rodrigues[1], Kayo Iywjhi de Lima Cantão Kawano,[4], Florentina D. Chilala[1], Hamilton S do Nascimento[1], Moyses dos Santos Miranda[1]

[1]Universidade Federal do Pará
[2]Instituto Federal do Pará
[3]Universidade Federal de São Paulo
[4]Faculdade Estácio de Sá de Castanhal
e-mail: lucianotstseguranca@gmail.com

In this assay, we evaluated the effects of exposing bovine embryos produced in vitro to moderate hypothermia (33ºC), aiming at the future study of cold shock proteins and the freezability of these embryos. Cumulus-oocyte complexes (COCs) from slaughterhouses were matured in vitro in TCM-199 supplemented with 10% FBS, 0.5 µg/mL FSH, 5 µg/mL LH, 11 µg/mL pyruvate and 10 µg/mL gentamicin for 22 hours. Frozen semen from a single bull was used, with spermatozoa separated by Percoll and co-incubated with the COCs (2x106 sptz/mL) in TALP-FERT medium for 18 hours. After IVF, the probable zygotes were cultured in vitro in drops of 40µL of SOF culture medium supplemented with 10% FBS, 6 mg/mL BSA. The embryos were cultured in normothermia (38.5ºC) until the 5th day of culture (IVF Day 0) when they were then exposed to moderate hypothermia (CO2 incubator adjusted to 33ºC) for 6 and 12 hours (cold-6 groups and cold-12, respectively) and then returned to normothermic culture. One group of embryos was maintained at all times under normothermic conditions (Control) and all groups were evaluated on the 7th and 8th day for the blastocyst formation rate and for the developmental kinetics of these formed embryos. Four replications were performed (n=402). The results were analyzed by ANOVA with Tukey's post-test, adopting a 5% significance level. We observed that culture in moderate hypothermia did not affect the rate of blastocyst formation on the 7th day (cold-6 = 24.9%, cold-12 = 22.0% and Control = 28.3%; $p>0.05$) and on the 8th day of cultivation (cold-6 = 30.1%, cold-12 = 26.2% and Control = 38.8%; $p>0.05$). However, regarding kinetics, we observed that exposure to hypothermia for 12 hours (cold-12 group) generated a greater number of initial blastocysts compared to cold-6 and control groups (78.0% vs. 63.7% and 59.4%, respectively; $p\leq0.05$) on the 7th day of cultivation. In addition, kinetic analysis on the 8th day revealed that the cold-12 group generated a lower proportion ($p\leq0.05$) of hatched blastocysts (20.4%) compared to the cold-6 (37.0%) and control (51.3%). Our results indicate that exposure of D5 embryos for 12 hours to moderate hypothermia did not affect the rate of blastocyst on the 7th day but delayed embryo development until the 8th day of culture. These preliminary results indicate that the cold-shock response metabolism may have been activated in the embryos and therefore the analysis of this mechanism needs to be performed to better understand the cold response mechanisms and the role of cold- shock proteins in this process.

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Cell-free DNA in bovine follicular fluid: a marker for oocyte competence

Nayara Kussano Ribeiro[1,2], Maurício Machaim Franco[3,2], Margot Alves Nunes Dode[4,5]

[1]Universidade de Brasília
[2]Embrapa Recursos Genéticos e Biotecnologia
[3]Universidade Federal de Uberlândia
[4]Universidade de Brasília (*Biologia Animal*)
[5]Embrapa Recursos Genéticos e Biotecnologia
e-mail: nayarakussano@hotmail.com

Oocyte quality is the central factor influencing the outcome of in vitro embryo production systems. However, oocyte quality evaluation is mainly limited to an assessment of morphological criteria. Therefore, there is a need to identify non-invasive markers to improve the accuracy of oocyte selection. This study aimed to evaluate if quantification of cell-free DNA (cfDNA) in bovine follicular fluid (FF) can be used as a marker for oocyte competence. To do that, 5-6mm follicles were dissected from ovaries collected from slaughterhouses. From each individual follicle, FF was collected and transferred to a 0.2 mL microtube, centrifuged at 300xg for 10 min at 4 °C to separate FF and cell fractions, supernatant (FF) transferred to another microtube, centrifuged again at 2000xg for 10 min and after centrifugation, supernatant was transferred to another microtube and centrifuged once more at 16500xg for 30 min at 4 °C, the supernatant was then individually stored at −80 °C.The oocytes were individually matured, fertilized and cultured until day 8 of development (D8). On D8, the collected samples of FF were grouped according to the result of embryo production into: FF in which the oocytes reached the blastocyst stage (EMB) and those in which oocytes cleaved but did not reach the blastocyst stage (NEMB). cfDNA was extracted from 30 individual samples of 15 µL of FF from each group (EMB and NEMB).The protocol was developed at the Laboratory of Animal Reproduction (Embrapa Genetic Resources and Biotechnology - Brasília- DF, Brazil) based on a salting out procedure. Then, the number of copies of ART2 and Bov-tA, that are short interspersed nuclear elements (SINEs), were used to quantify cfDNA in FF by qPCR. Due to the lack of studies in cattle quantifying cfDNA in FF, we based on reports in humans, which routinely use ALU sequences for cfDNA quantification. Considering that ALU was not found in ruminants, we searched for other SINEs that could be used in cattle. Thus, we choose to use ART2 and Bov-tA elements, which comprise ~11.91% of the bovine genome and are enriched in cfDNA. Data of the cfDNA quantification analyzed using the Mann–Whitney U test, GraphPad Prism 9 (GraphPad Software, San Diego, California USA). Results showed a greater amount ($P<0.05$) of cfDNA from the ART2 gene in the FF of the NEMB group compared to the EMB. It can be concluded that lower levels of the ART2 DNA in FF may indicate oocytes with greater potential for development.

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The contribution of lipids to the organelle differential profile of *in vitro*-produced bovine embryos

Kelly Annes[1], Christina Ramires Ferreira[2], Roniele Santana Valente[3], Thamiris Vieira Marsico[3], Juliana Hayashi Tannura[4], Juliano Coelho da Silveira[5], Flávio Henrique Silva[1], Fernanda da Cruz Landim-Alvarenga[6], Fernando Mesquita[7], Mateus José Sudano[1]

[1]Univerdade Federal de São Carlos
[2]Purdue University
[3]Universidade Federal do ABC
[4]Genus plc
[5]University of São Paulo
[6]Universidade Estadual Paulista
[7]Universidade Federal do Pampa
e-mail: keh.annes@gmail.com

An important feature that makes each living being unique is the lipid identity of its organelles (Gould et al., Curr Biol , 28:8, 2018). Furthermore, the diversity in the distribution of these molecules characterizes the role that each organelle plays in cellular activity (Casares et al., Int J Mol Sci 20:9, 2019). The characterization of the lipid profiles of whole embryos is well documented in the literature (Sudano et al., Biol Reprod 87:6, 2012). However, this type of approach can often lead to the loss of relevant information at the sub- cellular and consequently metabolic level, hindering the finer understanding of key physiological processes during preimplantation development. Therefore, we aimed to provide the characterization of four organelles from IVP bovine embryos: lipid droplets (LD), endoplasmic reticulum (ER), mitochondria (MIT), and nuclear membrane (NUC) and to evaluate the contribution of the lipid species to each organelle studied. Oocytes were IVM, IVF and IVC until D7 (medium ABS Pecplan®, Mogi Mirim). Expanded blastocysts (n=100 embryos/replicate; 3 replicates; grade 1) were submitted to cell organelle isolation, followed by the lipid extraction of cell organelles (Brettschneider et al., J Vis Exp 146, 2019) and lipid analysis by the Multiple Reaction Monitoring (MRM) profiling method (de Lima et al., Mass Spectrom 53:12 ,2018). The ion intensities of the surveyed MRMs were normalized by the total ion current (TIC) to obtain the relative ion intensities. The intensity values of each ion peak across multiple spectra were autoscaled (mean-centered and divided by the standard deviation of each variable) in order to give the same importance to every m/z value. The univariate (ANOVA followed by Fisher's test) and multivariate statistical models were performed using Metaboanalyst 5.0 (http://www. metaboanalyst.ca) by principal component analysis (PCA). LD and ER displayed a greater number of lipids (Phosphatidylcholine - PC, Ceramides - Cer and Sphingomielin - SM) with high signal-to-noise intensities, as they are organelles with a high rate of biosynthesis, lipid distribution, and storage and recycling of lipid species ($P<0.05$). NUC presented a more distinct lipid profile compared to the other three organelles with high relative intensities of PC, SM, and triacylglycerols (TG), which is consistent with its high nuclear activity ($P<0.05$). MIT has an intermediate profile, but it was close to LD and ER, which is in agreement with its autonomous metabolism for some classes of phospholipids (PL) ($P<0.05$). Our study was able to identify the lipid composition present in each organelle studied, and it was possible to associate the lipid roles with characteristic organellar activity. Our findings indicate which lipid species and classes are of relevance for the homeostasis and function of each associated organelles as well as provide tentative biomarkers for the determination of *in vitro* embryonic development and quality.

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In vitro maturation of bovine cumulus-oocytes complex in a reusable and reversible sealing microfluidic device

Franciele Flores Vit[1], Leonardo Ferraz Pereira dos SantoS[2], Juliano Rodrigues Sangalli[3,4], Lucimara Gaziola de la Torre[5], Felipe Perecin[3,4,6], Flávio Vieira Meirelles[4,6], Juliano Coelho da Silveira[7,4,8]

[1]Universidade de São Paulo
[2]Universidade de São Paulo
[3]Universidade de São Paulo
[4]Faculdade de Zootecnia e Engenharia de Alimentos - Universidade de São Paulo
[5]Universidade Estadual de Campinas
[6]University of São Paulo
[7]Universidade de São Paulo
[8]University of São Paulo
e-mail: ffvit@usp.br

The study of oocyte maturation is significant as it can provide answers about oocyte quality, which is the ability to be fertilized and to develop a healthy embryo. However, it is difficult to understand the oocyte maturation process individually on the macroscale. Furthermore, the macroscale culture system is static and does not correspond to what occurs in vivo. Thus, microfluidics can help to understand cell behavior in microenvironments and dynamic systems. This study aimed to compare maturation rates in plate and microfluidics culture systems. Firstly, we developed a reusable and reversible sealing microfluidic device in laminated polydimethylsiloxane (PDMSLAM), glass, and Polymethyl methacrylate (PMMA- acrylic). The cumulus-oocyte complexes (COC) from bovine ovaries were aspirated, and we selected COCs with one or two layers of cumulus cells and homogeneous cytoplasm. The COCs were matured in TCM119 B medium supplemented with 22 µg/ml sodium pyruvate, 50µg/ml gentamicin, 0.4% BSA, 10 ng/ml IGF-1 (insulin-like growth factor), 100ng/ml AREG, 10−2 IU /ml human recombinant FSH, 50 ng/ml 17β-estradiol, 150 ng/ml progesterone, and 25mM sodium bicarbonate. COCs were cultured in groups in plates and microfluidic devices (6 oocytes per droplet in plates and 3 COCs per chamber in a microfluidic device). The COCs cultured in plates were cultured with 40µL of medium (groups), and the oocytes cultured in microfluidic were cultured with 6µL of medium (group) with a constant flow rate of 1µL/ min during 24 h. We performed 6 routines with a pool of 100 COCs total (50 oocytes in plates-10 per drops-control and 50 in microfluidic device-2 per culture chamber). In addition, the microfluidic device allows collecting the maturation media in real-time for 24h. The media samples collected were centrifuged (3 serial centrifugations) and frozen at -80°C for future extracellular vesicle analysis. Statistical analyses of the 6 replicates were carried out by Student T-test. The maturation rate in microdevices and plates was compared regarding the presence of the polar body. The COCs cultured in a microfluidic system presented the first polar body at the end of the oocyte maturation process confirming the lack of adverse effects. The maturation mean rates in plates and microfluidic devices were 79%±1.41 and 79%±1,79 respectively and presented no statistical differences(p=0.132). Preliminary results show the potential of microfluidics for studying and understanding oocytes in vitro maturation since there was no difference in oocyte morphology and first polar body extrusion. In addition, the microdevice allows the recovery of COCs (oocytes), media collection in real-time, and the reuse of the microdevice. The results demonstrate that the material used for the construction of the microdevice does not interfere with the COCs maturation process, being an alternative for future studies of COCs and embryos in a less invasive way.

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Infrared spectroscopy with machine learning algorithm predicts pregnancy during TAI of bovine females

Willian Vaniel Alves dos Reis[1], Thiago Franca da Silva[1], Cicero Rafael Cena da Silva[1], Eliane Vianna da Costa e Silva[1], Alana Aparecida Amarilha Nobre[1], Gabrielle Terra Souza Netto[2], Gustavo Guerino Macedo[1]

[1]Universidade Federal de Mato Grosso do Sul
[2]Grupo Água Tirada
e-mail: willian.vet.ufms@gmail.com

The utilization of optical spectroscopy techniques has garnered significant attention due to their demonstrated potential in the precise and highly selective diagnosis of various diseases through the analysis of spectra obtained from biological samples. This study aimed to develop a rapid, accurate, and cost-effective methodology for detecting different fertility levels during the pre-breeding season and determining pregnancy status 30 days after insemination using biofluids from bovine females. Metabolites in the serum blood of Nellore heifers (n=90) with a BCS of 3, weighing 319±28 kg, exhibiting a cyclicity rate of 32.5%, and possessing a mean follicular diameter of 8.4±3.6 mm were characterized using optical spectroscopy techniques and machine learning algorithms. Blood samples were collected at three time points: at the initiation of the protocol (D0), upon removal of the P4 intravaginal device (D8), and on the day of pregnancy diagnosis (D40). After centrifugation at 600 g for 10 minutes, serum was aspirated and aliquoted into duplicate microtubes (1000 µL each), then stored at -80 ºC until further processing. Fourier Transform Infrared Spectroscopy (FTIR) with a resolution of 4 cm-1 and a range of 1800 to 900 cm-1, using a Perkin-Elmer Spectrum 100 equipment with an ATR accessory, was employed for serum analysis. The prominent double amide I vibrational bands around 1570 cm-1 were identified. Principal Component Analysis (PCA) evaluated clustering tendencies, while discriminative tests such as Support Vector Machine (SVM) and k-Nearest Neighbor (KNN) classified the samples. The Score plot derived from the first two Principal Components explained 92.3% of the data variance and clearly exhibited distinct cluster formations for the pregnant group. Adequate discriminative tests achieved pregnancy prediction accuracies above 70% at D8. These findings indicate the potential of the discrimination approach for prospecting females with a higher likelihood of becoming pregnant. The KNN algorithm exhibited a classification power exceeding 85% at D40 for pregnancy diagnosis, successfully distinguishing between pregnant and non-pregnant animals. The serum metabolome displayed discernible differences between animals with high and low fertility and those that became pregnant or not, enabling the development of distinct management strategies for different breeding seasons. In summary, the methodology demonstrated a rapid and cost-effective approach, enabling the prediction of fertility before TAI protocols and serving as a non-invasive tool for pregnancy diagnosis. This allows for the stratification of animals with varying fertility levels towards different objectives before the breeding season. Furthermore, considering a machine learning algorithm, running more samples over time improves the accuracy of pregnancy diagnosis, potentially replacing invasive methods such as rectal ultrasound in the future.

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THEMATIC SECTION: 36th ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

SUPPORT BIOTECHNOLOGIES: CRYOPRESERVATION AND CRYOBIOLOGY, DIAGNOSIS THROUGH IMAGING, MOLECULAR BIOLOGY, AND "OMICS

Investigating the metabolic signature of injectable multimineral based on organic and inorganic phosphorus, selenium, copper, magnesium and potassium in beef cows

Giovani Batista Pastre[1,2], Edson Guimarães Lo Turco[3], Guilherme Felipe Ferreira dos Santos[4,2], Bruna Lima Chechin Catussi[5], Arnaldo Gonçalves Soares Neto[6], Bruno Sivieri de Lima[7], Luc Durel[8], Pietro Sampaio Baruselli[9]

[1]Virbac Brasil, São Paulo
[2]University of São Paulo
[3]IonMedicine clinical metabolomics
[4]Virbac Brasil, São Paulo
[5]Departamento de Reprodução Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo
[6]Virbac Brasil, São Paulo
[7]Virbac Brasil, São Paulo
[8]Virbac SA, Carros, France
[9]Universidade de São Paulo
e-mail: giovani.pastre@virbac.com.br

The aim of this study was to evaluate the metabolic profile of beef cows treated with injectable multimineral based on organic and inorganic phosphorus, selenium, copper, magnesium and potassium (Fosfosal®). The experiment was developed in one beef farm located in Agua Clara, Mato Grosso do Sul state, Brazil. The primiparous Nelore cows received a protocol based on estradiol, progesterone and eCG on average 40 days after calving. A total of 72 Nelore (Bos indicus) primiparous were assigned to either one of the treatments on D0 of FTAI protocol: 1) Control (no treatment; n=24), 2) Group 10mL2X (treated with 10mL of Fosfosal® on D0 and 10 mL on D8, n=24) and 3) Group 15mL1X (treated with 15mL of Fosfosal® on D0, n=24). On D0 (beginning of FTAI protocol), D10, D21 and D39, blood samples were collected for metabolomics analysis. The metabolites were evaluated using targeted LC-MS/MS analysis in tandem method by triple quadrupole mass spectrometer. Biomarker analyses based on receiver operating characteristic (ROC) curves were performed by MetaboAnalyst 5.0. The top significant features were selected for Over Representation Analysis. MSEA or Metabolite Set Enrichment Analysis was used to identify biologically meaningful patterns that are significantly enriched in quantitative metabolomic data. Data were analyzed by Multivariate Variance Analysis (MANOVA) considering the time (day of blood collection) and doses (10mL2X or 15mL1X; probability < 0.05) and post-hoc analysis (Tukey HSD) were performed to determine the differences between groups. Pathways analysis revealed that injectable multimineral improved (P<0.05) the mitochondrial function (Mitochondrial Beta-Oxidation of short and long chain saturated fatty acids) and improved phospholipid biosynthesis. The significant metabolites related to the pathways analysis are: Propionyl-L-carnitine, Butyryl-L-carnitine, Hexanoyl-L-carnitine, Octanoyl-L-carnitine, O-Decanoyl-L-carnitine, Lauroyl-L-carnitine, Tetradecanoylcarnitine, LPC (17:1), LysoPC (16:0), Palmitoil-L-Carnitine, Stearoyl-L-carnitine, LysoPC (18:0) and PC (13:0/13:0). Changes in these metabolic pathways can improve cellular respiration, energy production and cell membrane phospholipid synthesis, stimulating cell development. Also, phospholipids have long been known to be critical components of various cellular processes. However, no changes in metabolic pathways were observed between treatment doses. In conclusion, these results suggest changes in metabolic pathways in cows treated with injectable mineral during the FTAI protocol. The treatment with Fosfosal® improved mitochondrial activity and phospholipid biosynthesis, which can improve cell function and protection against oxidative stress.

Animal Reproduction

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Effect of alpha-lipoic acid (ALA) supplementation during *in vitro* culture of vitrified ovine ovarian tissue

Lucy Vanessa Sulca Ñaupas[1,2], Francisco Denilson Rodrigues Gomes[1,2], Everton Pimentel Ferreira Lopes[3,2], Gildas Mbemya Tetaping[1,2], Anna Clara Accioly Ferreira[1,2], Ana Paula Ribeiro Rodrigues[1,2]

[1]Universidade Estadual do Ceará
[2]Laboratory of Manipulation of Oocytes and Ovarian Pre-Antral Follicles
[3]Universidade Estadual do Ceará
e-mail: lucnaupas@gmail.com

The objective of this work was to evaluate the effect of adding constant (100 µM/14 days) or sequential (50 µM/day 0-7 and 100 µM/day 8-14) concentrations of the antioxidant ALA in the culture medium of vitrified ovine ovarian tissue. Therefore, the ovaries of five crossbred adult ewes (4 - 6 years old) were collected, then the ovarian cortex of each pair of ovaries was fragmented into eight pieces and randomly divided into two groups: fresh (n=4) and vitrified (n=4). One of the fresh fragments was immediately fixed as a control, while the other three were cultured in vitro for 14 days under one of three conditions: (1) no ALA, (2) constant concentration, or (3) sequential concentration, using as base medium α-MEM at 7.5% CO2 and 38.5°C (Silva et al., Frontier in Veterinary Science, 9:822367, 2022). The vitrification was performed using the Ovarian Tissue Cryosistem device (Ñaupas et al., Livestock Science, 266:105123, 2022) and after warming, one fragment was fixed, and the other three were cultured under the same conditions as the fresh group. Fragments from all experimental conditions were fixed in Davidson's solution for 12 hours and stained with Periodic Acid-Schiff for evaluation of morphology and follicular development. Measurements of estradiol levels using an enzyme-linked fluorescent assay (ELFA) and the antioxidant capacity on free radicals 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) using enzyme-linked immunosorbent assay (ELISA) was performed on the culture media recovered on days 2, 8, and 14. Data were analyzed by one-way ANOVA and presented as mean (± SEM) or percentage, being considered significant when $P<0.05$. The results showed that the fresh ovarian tissue maintained a percentage of normal follicles similar to the control after 14 days of culture ($P>0.05$). In contrast, the tissue cultured after vitrification showed a significant reduction ($P<0.05$), with the most affected being those cultured in the absence of ALA. Cultured treatments, fresh and vitrified, showed follicular development. With regard to estradiol levels, they were detected during the first week of culture in all treatments. However, from day 8, only fresh and vitrified ovarian tissue cultured without ALA and with sequential ALA, respectively, remained unchanged (P>0, 05). Finally, the evaluation of antioxidant capacity showed that sequential ALA supplementation significantly reduced ($P<0.05$) levels of ABTS and DPPH radicals in fresh and vitrified cultures. In conclusion, the addition of an antioxidant during in vitro culture of vitrified ovarian tissue should be standard practice and the use of sequential ALA showed the best results in maintaining estradiol levels and controlling oxidative stress. However, the percentage of normal follicles showed a reduction regardless of ALA concentration.