Animal Reproduction

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

OPU AND IVF

Differential oocyte recovery rates after ovum pick-up and *in vitro* embryo production in Charolais and Hereford cattle breeds

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Selected purebred beef cattle present several advantages such as consistent, similar growth rates, similar weight, and carcass; however, these traits vary among breeds. The objective was to assess the differential efficiency of the ovum pick-up (OPU) technique in beef cattle purebreds evaluating the oocyte recovery rates and in vitro embryo production. A total of 48 OPU sessions were carried out in two Bos taurus beef cattle breeds [Hereford (H): 24 and Charolais (C): 24; Age: 3-7 y.o.; body condition score: 3.0-3.5] maintained under the same nutritional and management conditions. In vitro embryo production procedure was performed using conventional unsorted semen. All individuals were superstimulated as follows: Day 0: intravaginal CIDR (1.38g/ cow) + intramuscular progesterone (100 mg/cow) + intramuscular 17-beta-estradiol (2 mg/cow); Day 1: Intramuscular eCG (2500 IU/cow); Day 4: OPU. Different oocyte-derived parameters were evaluated: oocyte yield (OY) and quality (grade classification according to Stojkovic et al., Biol. Reprod, 64:904–909, 2001), oocyte/donor (OD), oocyte maturation (OM, based on COCs expansion, perivitelline space, 1st polar body extrusion, cytoplasmic color, and zona pellucida shape), OM/donor (OMD). In addition, embryo-derived parameters were assessed: cleaved/OM (CLOM), cleaved- embryos/donor (CLD), total embryos (TE), TE/donor (TED), TE/GI-II (TEG), TE/OM (TEOM), degenerated embryos (DE), and DE/donor (DED). Statistics were performed using GLMM (SPSS® 25, IBM Corp., USA). Differences were observed in OM (25.25±4.31 vs. 38.84±9.51), OD (18.40±3.87 vs. 27.25±6.84), GI-II (22.50±4.47 vs. 26.37±2.10), GIII (7.37±1.78 vs. 15.46±6.65), CLD (13.82±3.13 vs. 25.23±5.88), TED (4.54±1.12 vs. 8.25±2.39), TEG (0.25±0.04 vs. 0.30±0.03), DE (0.50±0.20 vs. 0.60±0.10), DED (0.43±0.26 vs. 1.74±0.44) when both breeds were compared being greater in C breed in all traits ($P \leq 0.05$). In conclusion, based on the obtained results regarding breed comparison overall the Charolais breed obtained greater records in most of the oocyte- and embryo-derived traits. It is worth noting that the number of viable oocytes and embryos obtained per donor after IVF was greater in Charolais compared to the Hereford breed.

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OPU AND IVF

Oocyte recovery rates after ovum pick-up in dual-purpose Bos taurus cattle breeds: effects of unsorted and sex-sorted semen on *in vitro* embryo production

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Dual-purpose cattle breeds present several advantages in many traits such as health, fertility, meat quality, and longevity over other breeds. The main aim was to evaluate the potential efficiency of the ovum pick-up (OPU) technique in dual-purpose cattle breeds assessing the oocyte recovery rates and in vitro embryo production using unsorted and sex- sorted sperm. A total of 160 OPU sessions were performed in two dual-purpose Bos taurus cattle breeds [Brown Swiss (BS): 40 and Swiss Fleckvieh (SF): 120; Age: 4-8 y.o.; body condition score: 3.0-3.5] maintained under the same nutritional and management conditions. *In vitro* embryo production procedure was performed using unsorted and sex- sorted semen. Four experimental groups were randomly formed: Brown Swiss-unsorted (BSU; n = 12), Brown Swiss-sorted (BSS; n= 28), Swiss Fleckvieh -unsorted (SFU; n= 64), and Swiss Fleckvieh -sorted (SF n= 56). All individuals were super-stimulated as follows: Day 0: intravaginal CIDR (1.38g/ cow) + intramuscular progesterone (100 mg/cow) + intramuscular 17-beta-estradiol (2 mg/cow); Day 1: Intramuscular eCG (2500 IU/cow); Day 4: OPU. Different oocyte-derived parameters were evaluated: oocyte yield (OY) and quality (GI-II-III), oocyte/donor (OD), oocyte maturation (OM, based on COCs expansion, perivitelline space, 1st polar body extrusion, cytoplasmic color, and zona pellucida shape), OM/donor (OMD). In addition, embryo-derived parameters were assessed: cleaved- embryos/OM (CLOM), cleaved-embryos/donor (CLD), total embryos (TE), TE/donor (TED), TE/GI-II (TEG), TE/OM (TEOM), degenerated embryos (DE), and DE/donor (DED). Statistics were performed using GLMM (SPSS® 25, IBM Corp., USA). Differences were observed in GIII (10.36±2.71 vs. 4.50±1.53), CLD (10.10±1.20 vs. 8.06±0.71), TED (3.74±0.76 vs. 1.79±0.41), TEG (3.50±0.73 vs. 1.60±0.21), TEOM (4.41±0.97 vs. 2.35±0.43) when both breeds were compared being greater in SF ($P \leq 0.05$). Differences were detected when unsorted and sex-sorted were compared in CLOM (4.70±0.68 vs. 2.39±0.20) and DE (4.70±0.68 vs. 2.39±0.20) ($P < 0.05$). Although embryo-derived parameters were lower in BSS and SFS groups, no differences were observed among all groups (BSU, BSS, SFU, and SFS; $p > 0.05$). No differences were observed among all groups regarding TED (BSU: 6.81±2.11; BSS: 3.00±2.52; SFU: 7.04±2.70; SFS: 3.32±2.10; $P > 0.05$). In conclusion, overall the SF breed obtained higher records in most of the oocyte- and embryo-derived parameters specifically, SF showed superior performance in oocyte-derived rates compared to the BS breed. The number of CLOM and DE was greater when using unsorted semen in the IVF. Finally, based on the obtained results regarding breed-semen interaction, all groups showed similar performance on embryo production rates.

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OPU AND IVF

Intrafollicular transfer of immature oocytes (IFOT): effect of the injection conditions on oocyte recovery and quality

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Despite being a very promising technique, the efficiency of Intrafollicular oocyte Transfer (IFOT) is still low. This study evaluated the best injection conditions to increase oocyte recovery, maintaining its quality. Four different needles (27G Gingival DFL®, Rio de Janeiro - Brazil (27G G); 27G WTA®, Cravinhos - Brazil (27G W); 27G Spinal Unisis®, Fukuoka – Japan (27G S); 30G Gingival DFL®, Rio de Janeiro - Brazil (30G G), three volumes of PBS medium (10, 20 and 60 µl) and two quantity (25 and 50) of cumulus-oocyte complexes (COCs) were evaluated. Ovaries were collected at slaughterhouses and follicles >9 mm were used for the experiments. At the time of injection, we evaluated resistance to penetrate the follicle (1 – the needle penetrates easily; 2 – moderate effort; 3 – higher effort), reflux from the orifice caused by the injection (1 – tiny drop; 2 – big drop; 3 - jet of liquid) and if COCs were retained in the system (% retention). Then, injected follicles were aspirated and the COCs recovery and denudation rates were evaluated. Data were analyzed by analysis of variance (SAS, 9.4 Version) and means compared by the probability of difference (PDIFF). First, we compared the four needles using 20 µl and 25 COCs, in 5 replicates. The retention rate was similar among all the needles (P>0.05). A greater resistance was observed in 27G S than in the other needles (P<0.05). Reflux was higher (P<0.05) in 30G G (3.0) than in 27G G (1.4) and 27G S (1.8), while 27G W (2.6) was similar to others. Recovery rate was lower (P<0.05) in 30G G needle (63.5%) and didn't differ among the others (27G S - 83.2%, 27G W - 82.5%, and 27G G - 79%). The 30G G (84.0%) and 27G G (66.5%) had higher (P<0.05) denudation rates than 27G S (27.0%) and 27G W (34.1%). Considering that 27G G and 30G G had the highest denudation rates, they failed to maintain the integrity of COCs and were excluded from the next experiment. Then, 10 replicates were performed using 27G S and 27G W to evaluate the effect of volume of medium (10, 20, and 60 µl), using 25 COCs. When only the effect of the volume was considered, the retention rate was higher (P<0.05) in 10 µl (8.24%) than in 20 µl (1.99%) and 60 µl (1.24%). However, no interaction between volume and needles was detected (P>0.05) for any variable. To evaluate the effect of the number of COCs, we compared 25 and 50 COCs, using 10 or 20 µl and 27G S and 27G W, in 9 replicates. The results showed that when 27G S was used, there was an effect of the volume on the recovery rate (P<0.05), being higher in 10 µl (85.82%) than in 20 µl (70.05%). In addition, there was an effect of the interaction among needles, volume, and quantity of COCs for the retention rate (P<0.05). The highest retention rate was when 27G W was used with 25 COCs in 10 µl. It can be concluded that a 27G S needle with 10 µl is the most suitable to be used in IFOT since it has the highest recovery rates, maintaining COCs quality.

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

OPU AND IVF

Effect of treatment with injectable mineral supplementation on the efficiency of *in vitro* embryo production in Holstein heifers

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This study aimed to assess the impact of injections of a commercial multi-mineral solution (inorganic phosphorus, organic phosphorus, selenium, magnesium, copper, and potassium) on follicular aspiration (OPU) results and *in vitro* embryo production (IVEP) in Holstein heifers. Eighty heifers were divided into two experimental groups: 1) Control Group - 40 heifers did not receive the mineral supplement, and 2) F Group - 40 heifers received 10 mL/animal i.m. of Fosfosal® (Virbac, Brazil) on D-30 (thirty days before D0) and on D0 (D0 = start of protocol). All donors were submitted to a protocol to synchronize a new follicular wave. On a random day of the estrous cycle (D0), females received an intravaginal P4 device (PRIMER®; Agener União) and 2 mg of estradiol benzoate (E2; RIC BE®; Agener União) and 0.530 mg of sodium cloprostenol (PGF; Estron®; Agener União) i.m. Seven days later (D7), the device was removed and OPU was performed. The aspirations were always conducted by the same technician and the embryos were produced in the same laboratory using the same batch of semen. The number and quality of oocytes recovered, the cleavage rate, and blastocyst production were evaluated. The number of recovered oocytes (11.7±0.88 vs. 15.6±1.34; P = 0.02) was significantly higher in group F, as well as the number of viable oocytes (6.1±0.47 vs. 8.8±0.81; P = 0.006) and the number of cleaved oocytes (4.67±0.52 vs. 6.62±0.67; P = 0.02). Furthermore, the cleavage rate tended to be higher in group F (37.0±0.03% vs. 42.5±0.03%; P = 0.07). No significant differences were observed between groups regarding the number of blastocysts and the rate of blastocysts produced over the total number of oocytes retrieved. These results suggest that intramuscular mineral supplementation with Fosfosal® can increase the quantity and quality of oocytes retrieved from Holstein heifers submitted to OPU/IVEP.

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OPU AND IVF

Fertilization in the presence of oviduct epithelium fragments increases interferon tau expression on bovine *in vitro* embryos production

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The uterine tube is known to provide the ideal conditions for fertilization and initial embryonic development. One of the main challenges of IVP is precisely to provide more adequate conditions during the interaction between male and female gametes. The objective of this study was to evaluate the effect of adding oviduct epithelium fragments (OEF) during the process of fertilization in bovine. For each replicate, uterine tubes were collected from non-pregnant animals in metaestrus. In the laboratory, the region corresponding to the ampulla was sectioned and, with the aid of a scalpel, was compressed in the direction from the isthmus to the infundibulum. The fragments were transferred to a minitube with 1 mL of Medium 199 [supplemented with 10% SFB and gentamicin], and subjected to continuous pipetting to reduce the of the fragments and then centrifuged for 5 seconds (600g). The pellet was resuspended in 1 mL of Medium 199 and centrifuged again. The pellet formed was homogenized and 7 µL of OEF were deposited into 100 µL drops of IVM (Medium 199 supplemented with 10% SFB, FSH, hCG, pyruvate and gentamicin) and kept in culture for 18 h aiming to condition and synchronize for fertilization. Six replicates were performed, a total of 271 matured COCs were randomly assigned between Control Group (142 COCs) and OEF Group (129 COCs; fertilization in the presence of oviduct epithelium fragments). IVF was performed in 100 µL drops of TALP medium plus BSA, penicillamine, hypotaurine, epinephrine, Heparin, pyruvate, and gentamicin. Twenty-four hours after IVF, the presumptive zygotes were transferred to 100 µL drops of SOF medium supplemented with 10% SFB, glucose, BSA, pyruvate and gentamicin) where they remained for 8 days before being evaluated and stored for gene expression analysis. Cleavage rates (day 2), blastocyst formation (day 8) and embryonic gene expression results were subjected to ANOVA (Tukey's post-test, $p<0.05$). RT-PCR was done for gene expression analysis (3 replicates with a pool of 5 embryos each), using the method of $2\Delta\Delta CT$ and with YWHAZ being an endogenous gene. There was no significant difference ($p>0.05$) occurring in cleavage rates (90.7% vs. 88.5%) nor blastocyst formation (40.9% vs. 51.5%) between Control Group and OEF Group, respectively. Analysis of the relative expression of genes related to cellular protection against oxidative and thermal stress revealed no difference between the experimental groups for SOD (P=0.988) and HSP70 (p=0.782), respectively, nor for OCT-4 (p=0.721) an important regulator of transcription during early embryonic development and cell differentiation. However, there was higher expression (P=0.003) in the OEF Group of IFNT, a protein produced by the embryonic trophoblast and essential for maternal recognition of pregnancy and which is highly correlated with embryo quality. These results suggest that the addition of OEF during the *in vitro* fertilization process may increase the quality of embryos produced.

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OPU AND IVF

Total medium renewal with changing fetal bovine serum concentration during *in vitro* culture of bovine embryos reduces stress and increases cell pluripotency

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The objective of this work was to evaluate the effect of the total renewal of the medium, as well as the influence of the FBS reduction at the beginning of the culture on the rates of blastocyst formation, embryonic kinetics, and expression of marker genes of embryonic quality (OCT4, IFN-τ, SOD, HSP70 and BAX). For this purpose, the zygotes were distributed into the following experimental groups: G1 (n=452) – the embryos remained in the same drop during the 8 days of culture containing 10% FBS G2 (n=142) – the embryos were exchanged 72 hours after the start of IVC, maintaining the concentration of 10% FBS G3 (n=209) – the embryos were cultured for 72 hours in medium containing 5% FBS and then transferred to another drop of medium with 10% FBS. Cumulus oocyte complexes (COCs) were obtained from puncture of ovaries collected in a slaughterhouse and IVM (TCM 199 supplemented with sodium bicarbonate, FSH, LH, pyruvate, antibiotics and 10% FBS) for 18 hours. For fertilization, the COCs were co-incubated with spermatozoa for 24 hours and the zygotes were cultivated in SOF medium (glucose, BSA, pyruvate e gentamicin) according to the experimental groups described above. All steps took place in an incubator with 5% CO_2, 20% O_2 and 75% N_2 under a humid atmosphere and temperature of 38.5°C. The rate of blastocyst formation and embryonic development kinetics (evaluation of embryonic stage) was evaluated on D7. Embryo gene expression was evaluated by real time PCR (three replicates with a pool of five embryos each) using the StepOne plus® system (Applied Biosystems©) and statistical analysis was performed using SigmaPlot v. 12.0, using ANOVA with a significance level of 5%, and applying the Holm-Sidack post-test when necessary. No significant differences were observed in the rates of blastocyst formation (P = 0.859) (G1 - 39,287±9,053; G2 - 38,121±6,599; G3 - 40,323±6,540) or in the kinetics of embryonic development (P >0.05) between the experimental groups. In the gene expression analysis, significant differences were observed between the experimental groups in three of the evaluated genes. There was a reduction in IFNT τ expression (P = 0.001) in the G3 group (0.0919±0.0217) compared to the other groups (G1 0.549±0.214 and G2 0.416±0.140), probably due to the reduction in the FBS concentration in the first hours of cultivation. Regarding OCT4 (P = 0.010), the G3 group showed a high expression compared to the G1 group (1.003 ± 0.251 and 0.689 ± 0.259, respectively); with the HSP70 gene, cell stress response gene, groups G2 (0.337±0.252) and G3 (0.359±0.177) showed lower relative expression (P = 0.001) compared to group G1 (1.072±0.565), demonstrating a beneficial effect on the quality of embryos that were submitted to the total medium renewal system. The total renewal of the culture medium and the alteration of the FBS concentration reduce the stress and increase the expression of pluripotency genes in the embryos produced *in vitro*.

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OPU AND IVF

Effects of the oocyte donor breed and season at the time of follicular aspiration on IVP outcomes in cattle.

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In vitro embryo production (IVP) is a reproductive technique that aims to accelerate the genetic gain of herds by the male and female multiplication. However, the success of this technique depends on several factors. The aim of this study was to evaluate the effects of the animal breed (Gyr or Girolando) and the period of the year (dry period = autumn/winter or rainy period = spring/summer) at the time of follicular aspiration (OPU) upon productivity parameters such as: oocyte production, blastocyst production and pregnancy rate of recipients. The database of 1198 OPU, 27135 oocytes and 4483 ET from a commercial laboratory of embryo production, located at Triângulo Mineiro, Minas Gerais, Brazil was analyzed. The number of viable oocytes and embryos produced were evaluated by ANOVA and the pregnancy rate of recipients was evaluated by logistic regression, both in MINITAB program, including in the model the donor breed and season of the year at the OPU, as well as the interactions. The effect of the interaction between donor breed and period of the year at OPU ($P<0.01$) was observed on the number of collected oocytes and embryos produced. During rainy season the production of oocytes and embryos were no different for Gyr (24.10 ± 0.971; 7.18 ± 0.406 and Girolando (24.54 ± 0.859; 7.50 ± 0.462), however, in dry season, Girolando donors had higher oocyte and embryo production (23.58 ± 0.789; 8.85 ± 0.425) compared to Gyr (19.43 ± 0.792; 5.75 ± 0.283). The embryo production rate was neither affected by the period of the year at the OPU nor by the breed of the donor, ranging from 29.20 to 35.50%. There was no interaction between donor breed and period of the year at OPU on pregnancy/ET. However, donor breed affected pregnancy/ET, with Gyr donors being superior (49.18% x 44.13%; $P<0.05$), although no effect of the period of the year at OPU was observed in P/ET (46.64% dry period x 45.92% rainy period). In conclusion, the Girolando donor produced a higher number of viable oocytes and embryos per aspiration at the dry period, and the Gyr embryos resulted in higher pregnancy/ET in any period of the year.

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OPU AND IVF

Chromatin compaction and transcriptional activity in oocytes recovered from early antral follicles and cultured in vitro with Trichostatin A

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Trichostatin A (TSA) promotes histone hyperacetylation and has been used during in vitro culture of oocytes in order to increase their transcriptional activity. In order to establish a culture system capable of increasing the accumulation of transcripts, aiming to improve the acquisition of oocyte competence, oocytes retrieved from antral follicles <2 mm in diameter were pre-matured (PM) in vitro with the meiotic blocker C-type natriuretic peptide (NPPC) associated to TSA. The follicles present in the cortical portion of the ovary were measured with a ruler attached to the stereoscope and those with a diameter <2 mm were ruptured for recovery of COCs, which were PM for 24h in 100 µL microdrops of TCM-199 with 0.2 mM pyruvate, 25 mM sodium bicarbonate, 75 µg/mL amikacin, 0.3% BSA, 1x10-4UI/mL rFSH, 100 nM NPPC and different concentrations of TSA (0 nM; 2.5 nM; 5.0 nM and 10.0 nM). Next, the oocytes were IVM (TCM199 with 1x10-1 UI/mL rFSH, 100 UI/mL hCG and 10% FBS) for 24h. Immature oocytes were also evaluated immediately after removal from the follicle (group 0h). At the end of each moment, the COCs were stripped from cumulus cells and their diameter was measured. To assess chromatin compaction, the oocytes were stained with Hoechst 33342; immature oocytes (germinal vesicle - GV) were classified from GV0 to GV3 (from least to most compact chromatin) and oocytes with contracted metaphase chromosomes and a polar body were classified as metaphase II (MII). The global transcription activity was assessed after staining with Click-it RNA Imaging Kit. The images were evaluated in an epifluorescence microscope to determine the arbitrary units of fluorescence (AUF). Data were analyzed by analysis of variance (ANOVA) followed by Tukey's test ($P<0.05$). At the end of the PM, there was no difference between the groups regarding the oocyte diameter (101.9 to 104.3 µm; $P>0.05$), but there was an increase ($P<0.05$) in the diameter of the oocytes at the end of IVM (106.9 to 109.1 µm, regardless of the presence of TSA) compared to immature oocytes (0h: 102.6 µm). At the end of PM and IVM, most oocytes were still under meiosis blockage and chromatin compaction was evenly distributed in the GV phases ($P>0.05$). The rate of oocytes that reached MII ranged from 11.8% to 18.0%, with no difference between groups ($P>0.05$). The fact that the oocytes were unable to complete their growth may explain why they did not acquire meiotic competence. The transcriptional activity of oocytes in the GV0 phase at the end of PM was higher ($P<0.05$) in TSA10 group (32.4±1.7 AUF) compared to TSA5 (22.1±2.3 AUF), TSA2.5 (16.8±1.2 AUF) and control (20.3±2.9 AUF). We conclude that TSA at 10.0 nM was efficient to promote the increase in transcriptional activity when associated with NPPC during the PM of oocytes recovered from follicles <2 mm, however, these oocytes were not able to complete the growth and to acquire the meiotic competence.

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OPU AND IVF

Lipid modulation during IVM in the cat model: role on oocyte lipid content, nuclear maturation, oxidative stress, mitochondrial activity, gene expression, and cryopreservation

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The domestic cat is a useful model for assisted reproduction techniques aiming for the application in endangered wild cats. The amount and composition of intracellular lipids are important factors in oocyte viability during cryopreservation. Feline oocytes present a high concentration of intracellular lipids, and although they present important cellular functions, studies indicate that lipid content increases during IVM. This study assessed the role of lipid modulators added during IVM on lipid content, nuclear maturation, oxidative stress, mitochondrial activity, gene expression, and cryosurvival of cat oocytes. The COCs were recovered from ovaries obtained in elective surgeries and selected based on cytoplasm homogeneity and cumulus cell layers (a total of 20 replicates of COC selection and IVM, n=705). First, IVM (TCM 199 supplemented with 0.02 IU/mL FSH/LH, 100 µM cysteamine, 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 0.055 mg/mL gentamicin, for 28 h at 38.5 °C in maximum humidity) was performed comparing three lipid modulators, besides the control group (CONT): 100 µM conjugated linoleic acid (LA), 100 µM forskolin (FK) and 0.5 mg/mL L-carnitine (LC), and the lipid content (Oil Red staining) was assessed. Then, the LA was compared with a MIX of the three modulators (LA+FK+LC) on lipid content. Subsequently, both CONT and MIX were compared regarding nuclear maturation (Hoechst 33342). After that, oocytes from CONT and MIX groups were submitted to mitochondrial activity, reactive oxygen species (ROS), and glutathione (GSH) levels analyses; to the relative expression of *SDHA, GDF9, BMP15, ZAR-1, PRDX1, SIRT1*, and *SIRT3* genes (normalized by *ACTB* and *YWHAZ* genes); and to vitrification and viability assessment (Neutral Red Staining). Regarding lipid content, the LA presented a reduced ($P<0.05$) lipid content compared to CONT and FK, while the other groups had no difference ($P>0.05$). Then, the MIX showed a significant ($P<0.05$) reduction in oocyte lipid content, in comparison with LA. No difference ($P>0.05$) was observed in the MII rate in the CONT (45%) and MIX (41%) groups and in mitochondrial activity Interestingly, although ROS and GSH levels were higher ($P<0.05$) in MIX than in CONT, the redox balance (ROS/GSH) was greater ($P<0.05$) in the latter. The *GDF9, PRDX1*, and *SIRT1* genes were downregulated ($P<0.05$) and *BMP15* presented a tendency ($P=0.056$) to downregulation in MIX. After vitrification, MIX (74%) presented a higher ($P<0.05$) viability compared to CONT (53%). It was concluded that although not influencing the MII rate, the MIX complex, in addition to reducing the total lipid content of IVM-oocytes, seems to improve viability after cryopreservation and to affect the oocyte metabolism, and such changes still needs to be better understood in the cat biological model.

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Animal Reproduction

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

OPU AND IVF

Resveratrol supplementation does not improve the *in vitro* nuclear maturation rate of feline oocytes

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Domestic cats are seen as an important experimental model for the application of reproductive biotechnologies in endangered wild cats, by improving the knowledge of reproductive physiology. Oocyte *in vitro* maturation (IVM) is a fundamental step for *in vitro* embryo production (IVP). One of the challenges in IVM efficiency are the reactive oxygen species generated during the process. The aim of this study was to evaluate the antioxidant action of resveratrol in two different moments: either in a previous exposure before IVM or during the IVM of feline oocytes. For reaching that, oocytes were recovered from feline ovaries obtained in elective ovariosalpingohisterectomy surgeries and selected when presenting homogeneous cytoplasm and, at least, two layers of cumulus cells. Firstly, four replicates (n=139 COCs) of IVM were performed comparing the groups: control (CONT), in which oocytes were not pre-exposed to resveratrol (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); and pre-exposed (PRE), where oocytes were exposed to 0.2 µl/mL of resveratrol (TCM 199 HEPES, supplemented with 4 mg/mL of BSA, 50 µL/mL of pyruvate, 10 µL/mL of penicillin-streptomycin, 0.2 µl/mL of resveratrol) for 90 min, at 38,5 °C, in 5% O2, 5% CO2, and 90% N2 before IVM (same IVM media as CONT) under the same conditions. In addition, five replicates (n=222 COCs) were performed, comparing COCs allocated into two groups: control (CONT), oocytes with no exposure to resveratrol in IVM (same IVM media as the previous CONT); or exposed (EXP), in which oocytes were exposed to resveratrol during IVM (same IVM media as CONT with 0.1 µL/mL of resveratrol). All four groups remained in the IVM for 24 h, under the same temperature and atmosphere conditions described. After IVM, oocytes were denuded using hyaluronidase and gentle pipetting, fixed in 4% paraformol for 40 min, and stained with HOECHST 33342 for evaluation of the nuclear configuration under a fluorescence microscope. The Fisher's Exact Test was used to compare the groups, and $P<0.05$ was considered statistically significant. No differences ($P>0.05$) were observed in the nuclear maturation rates when resveratrol was tested previously to IVM [CONT (53,73%) and PRE (59.7%)] or during IVM [CONT (51.3%) and EXP (41.0%)]. It was concluded that resveratrol did not affect the efficiency of IVM in feline oocytes neither when used during IVM nor in a previous exposure.

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Animal Reproduction

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

OPU AND IVF

In vitro production of embryos from young Nelore (*Bos indicus*) donors

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In vitro embryo production (IVP) has been frequently used for the genetic improvement of animals of high zootechnical value. Furthermore, this technique allows prepubertal females to be multiplied, which reduces the generation interval and accelerates genetic gain. However, although often applied to adult donors, this technology has lower success when used in young donors. The present study analyzed data regarding the first follicular aspiration (OPU) of 220 Nelore donors aged between 5 and 24 months, which were generated over the years 2020/21 by Agropecuária Sino, located in Uberaba-MG. For categorization, donors were distributed in the following classes according to age (months): 5 to 7 (n=5), 8 (n=23), 9 (n=30), 10 (n=20), 11 (n=47), 12 (n=34), 13 (n=16), 14 to 16 (n=16), 17 to 19 (n=15) and 20 to 24 (n= 14). Data related to oocyte and embryo production were analyzed through linear regression by the SAS® PROC GLIMMIX software (9.4). Results were expressed as mean and standard error. The number of retrieved (38.2±1.72; P=0.35) and viable (32.1±1.54; P=0.49) oocytes per procedure and the rate of viable oocytes (82%±0.75; P=0.93) did not differ between the categories. However, the age of the donor influenced (P<0.0001) the embryo production per OPU (5 to 7m=3.8±1.82c; 8m=6.3±0.92c; 9m=5.9±0.99c; 10m=7.3±1.70bc; 11m=7.7±1.09bc; 12m=8.9±1.67bc; 13m=8.6±1.59bc; 14 to 16m=12.4±2.43a 17 to 19m=17.5±2.60a; 20 to 24m=15.3±3.57a). The embryo production rate per OPU was also influenced (P<0.0001) by the age of the donor (5 to 7m=20%±0.072bc; 8m=16.2%±0.019c; 9m=16.8%±0.022c; 10m=17.2%±0.023c; 11m=20.2%±0.019bc; 12m=23.2%±0.023bc; 13m=26.3%±0.041 14 to 16=31.8%±0.025a 17 to 19m=41.2%±0.033a; 20 to 24m=32.9%±0.037ab). In addition, a second analysis was performed to assess the effect of the donor's age at the first OPU on embryo production after four consecutive aspirations in the same donor. The number of embryos (P<0.0001) produced was lower in donors aged 9 months (n=58; 7.8±0.56 embryos) compared to donors aged 12 months (n=64; 11.6±0.64 embryos). It is concluded that young donors, from 5 to 9 months, have similar efficiency in the number of embryos produced by OPU until 13 months of age, when there is an increase in embryo production.

Animal Reproduction

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

OPU AND IVF

In vitro embryo production from Nelore oocyte donors immunized against GnRH and stimulated with pFSH or rbST: preliminary results

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Previous studies from our group demonstrated that follicular wave suppression by immunization against GnRH does not affect oocyte recovery and *in vitro* embryo production (IVEP) from cows with chronic cystic ovarian disease. The aim of this study was to evaluate immunization against GnRH, associated with ovarian priming with follicle stimulating hormone (FSH) or recombinant bovine somatotropin (rbST), as a strategy for the preparation of donors for IVEP. Nelore cows (*Bos indicus*, n=40) underwent OPU at a random day of estrous cycle (Day 0) and were allocated into four groups: G1, negative control group, no hormonal pre-treatment; G2, anti-GnRH vaccine at Days 0 and 14; G3, anti-GnRH vaccine at Days 0 and 14, and 500mg rbST at Day 30; G4, anti-GnRH vaccine at days 0 and 14, and 140mg FSH split into four shots given at Days 43 and 44. All cows underwent OPU at Days 30 and 45. Data were analyzed using the Glimmix procedure of the SAS. Results are shown as mean±SEM or %. The effectiveness of the active immunization against GnRH was already observed at the 2nd OPU session, with a reduction on both the percentage of medium (5 to 7 mm, 1.7% vs. 5.6%, P=0.0001) and large follicles (>7mm, 0.1% vs. 2.9%, P 0.0001) present in the ovaries, compared with the control (non-immunized) group. The treatment with FSH (G4) increased the percentage of medium plus large follicles (>5 mm), compared with the other groups (54.7% vs. 4.9%, 0.4% and 0.0% for G1, G2 and G3, respectively, P<0.0001). There was no difference among groups on the total number of follicles (P=0.8768), number of total and viable COC recovered (P=0.9409 and P=0.6446), cleavage rate (P=0.1289) or number of embryos produced (P=0.6265). However, embryo rate differed among treatments (29.2%a, 43.9%b, 30.1%a and 42.7%b for groups G1, G2, G3 and G4, respectively, P=0.0008). Interestingly, the effect of treatment was affected by total follicle population (AFC). In cows with lower AFC (16.2±1.8, ranked in quartiles 1 and 2), immunization decreased the number of better-quality COC (grades I and II), whereas this effect was reversed by the FSH (3.0±2.3, 0.5±0.3, 0.5±0.3 and 3.2±0.7 for G1, G2, G3 and G4, respectively, P=0.0123). This effect was not observed (P=0.3852) for those cows with higher AFC (47.7±3.7, ranked in quartiles 3 and 4). In summary, active immunization against GnRH does not affect oocyte yield and improves blastocyst rate. A single priming with rbST had no beneficial effect on IVEP, whereas the effect of FSH will vary according to the AFC of the donors.

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Animal Reproduction

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

OPU AND IVF

Improved blastocyst formation using culture medium with fructose

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The in vitro production of buffalo embryos (IVP) is in constant expansion but still needs improvements, especially in relation to the composition of the embryonic culture media since they are still based on bovine experiments. Consequently, issues such as the demands for ideal energetic substrates for the species remain unclear. In many species of mammals, glucose is the main supplier of energy in the pre-implantation phase. However, studies with alternative sources of energy support buffalo embryo culture and have provided excellent results. Thus, the present work aimed to evaluate the replacement of glucose with fructose during buffalo embryo development. For this, 222 cumulus oocyte complexes (COCs) from ovaries obtained from a local slaughterhouse were matured in vitro for 18 hours and fertilized using the semen of a single bull. After fertilization, the presumptive zygotes were randomly allocated into two groups for IVC with SOF medium, either supplemented with 1 mM glucose (122 COCs) or 1 mM fructose (100 COCs). The IVC lasted for 8 days, and the resulting embryos were stored for later expression of key genes in the pre-implantation period: *OCT-4*, *Interferon-tau*, *BAX*, and *SOD*. Statistical analysis was performed using Sigma Plot 12.0 software, ANOVA test with a significance level of 5%. A total of 5 replicates were developed resulting in 42 embryos from the fructose group and 34 from the control group, as well SOF fructose group showed a higher rate of blastocyst formation (n/COCs total) both on the 7th day (30.3% ± 3.3 vs 23.5% ± 4.6) and on the 8th day (34% ± 5 vs 27.7% ± 2) than the glucose group, respectively ($p < 0.05$). In terms of the kinetics of embryonic development, there were no differences in the number of hatched blastocysts between the fructose (12.4% ± 11.3) and glucose groups (27.5% ± 28.5) evaluated on the 7th day. Using glucose or fructose for embryo culture, we observed that there was no significant difference in gene expression for *OCT-4*, *Interferon-tau*, *BAX*, and *SOD*. The study concluded that 1mM fructose can significantly increase buffalo blastocyst formation and preserve embryonic quality, which proves to be an excellent alternative energy source for buffaloes.

Animal Reproduction

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

OPU AND IVF

Assessment of oocyte quality and *in vitro* embryo production of prepubertal Nellore heifers treated with injectable progesterone

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For the success of in vitro embryo production (IVEP) and high cleavage rates of oocytes, there are factors that may interfere, such as the age of the donor, nutritional status, and hormonal concentrations. Oocytes recovered during the estrous cycle phase with high plasma progesterone concentration have better quality when compared to oocytes recovered during other phases of the estrous cycle. The evaluation of IGFBP and CASPASE expression is one of the methods to estimate quality, apoptosis rate, follicular atresia, and viability of oocytes to be used for IVEP. Thus, the objective of the study was to evaluate if treatment with injectable progesterone 7 days before transvaginal ultrasound-guided oocyte retrieval (commonly called OPU) would improve the quality of oocytes from prepubertal Nellore heifers. The study was conducted at the Advanced Research and Development Center for Beef Cattle in Sertãozinho, SP. Twenty-three Nellore heifers, approximately 13±0.8 months old, were allocated into two groups; progesterone group (PG), with an injection i.m (1.0 mL) of 150 mg/mL injectable progesterone (Sincrogest® Ourofino, Cravinhos-SP) 7 days before OPU, and control group (CG), without progesterone injection. After 30 days, a second OPU was performed (crossover between heifer groups). After the OPU, the oocytes were selected for IVEP, and a sample was fixed in 4% paraformaldehyde. Viable oocytes were subjected to immunofluorescence staining for IGFBP2 and CASPASE-3. To evaluate IGFBP2, a pool of 83 oocytes from the PG and 107 oocytes from the CG were analyzed. For the evaluation of CASPASE-3, 78 oocytes from the PG and 105 from the CG were analyzed. Oocytes were permeabilized with 0.5% Triton X-100 for 30 min at room temperature and washed three times in PBS supplemented with 0.2% Tween-20 (PBS-T) for 10 min and examined under a fluorescence microscope. For each structure, an image was generated per channel (A555 and A488 filters), and analyzed with ImageJ software (NIH, USA) for quantification of fluorescence intensity (pixels). The data were analyzed by the SAS® MIXED procedure. There were no differences in pixel intensity between PG and CG in IGFBP2 levels (137.37 vs. 135.43; P=0.67, respectively) and CASPASE-3 (118.96 vs. 118.22; P=0.24, respectively). In this study, the treatment with injectable progesterone before OPU in prepubertal Nellore heifers did not improve oocyte quality.

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Animal Reproduction

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

OPU AND IVF

Expression of miRNAs regulatory of lipid metabolism genes in oocyte and cumulus cells immature and *in vitro* matured of bovine

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MicroRNAs (miRNAs) are small and conserved molecules that regulate the levels of specific targets of messenger RNA (mRNA) in a post-transcriptional manner. miRNA may cause the degradation or prevent translation of mRNAs in cytoplasm through the base-pairing mechanism and are reported to play an important role in oocyte maturation and metabolism. Therefore, herein we study the lipid metabolism in immature and in vitro matured oocyte and cumulus cells in cattle, through the quantification of transcripts of regulatory miRNAs and their respective target genes. COCs were recovered through follicular puncture of ovaries obtained from a local slaughterhouse. Part of the COCs were stored before maturation, thus remaining immature (n=90). Another part of the COCs were matured in vitro (n=60) in TCM 199 medium supplemented with 10% FBS at 37°C in an incubator with a humidified atmosphere of 5% CO2 for 22 hours. Both groups were processed to remove the cumulus cells using hyaluronidase and the zona pellucida using pronase, after which they were stored in RNA later and preserved at -80°C. The mRNA and miRNA extraction in oocytes and cumulus cells were performed using TRIzol LS® method, followed by quantitative reverse transcription PCR (qRT-PCR), in triplicate, for the relative quantification of the ACACA, CPT2, FABP3, FASN, PLIN2 and SCD genes. The online tools TargetScan, miRDB and miRBase were used to predict regulatory miRNAs for the target genes mentioned above and the following miRNAs were identified: miR-205, miR-124B, miR-27a, miR-27b and miR-142-5P. As endogenous genes, Let-7a and U6 were used for miRNAs, and GAPDH and β-actin were used for mRNA. As a result, in vitro mature oocytes showed increased expression of ACACA (11.84 vs. 2.42, P< 0.05), CPT2 (3.00 vs. 1.31, P< 0.05), FASN (0.19 vs. 0.06, P< 0.05), PLIN2 (32.82 vs. 18.05, P< 0.05) and SCD (0.55 vs. 0.13, P< 0.05) compared to immature oocytes. As for regulatory miRNAs, mir-142 expression was increased on *in vitro* matured oocytes (0.08 vs. 0.02, P< 0.05) while mir-27 was increased in immature oocytes (0.0002 vs. 0.0001, P< 0.05). In cumulus cells, FABP3 expression was 2.1 times increased in immature oocytes compared to in vitro matured ones (3.33 vs. 1.60, P< 0.05), while no difference was observed in the remaining genes and miRNAs (P> 0.05). These results suggest that mir-142 and mir-27a are related to lipid metabolism during oocyte maturation in vitro, specifically in fatty acid oxidation and lipogenesis pathways. Theoretically, mir-142 could interact with PLIN2, ACACA AND FASN to regulate the storage of free fatty acids as cytoplasmic lipid droplets in oocytes. We conclude that miRNAs are associated with changes in lipid metabolism in bovine COCs submitted to in vitro maturation.

Animal Reproduction

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

OPU AND IVF

Fertilization with follicular fluid reduces *HSP70* and *BAX* expression on bovine *in vitro* embryos

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The *in vivo* process of fertilization occurs in the presence of follicular fluid (FF), a blood plasma-derived liquid with a dynamic composition. This fluid is involved in the oocyte growth and maturation processes, as well as in sperm capacitation. The objective of this study was to evaluate the effect of fertilization medium supplementation with 5% or 10% FF on *in vitro* embryo production yield and on expression of marker genes of embryonic quality (OCT4, IFNT-tau, SOD, HSP70 and BAX). To this end, FF was collected from ovarian follicles with a diameter of 8mm to 10mm. After aspiration the FF was deposited in 15 mL tubes and submitted to centrifugations (600g) for 25 minutes, followed by filtration and storage at -20°C. Cumulus oophorus complexes (COCs) obtained from a local abattoir were selected and matured *in vitro* for 18h. For fertilization, the COCs were co-incubated with spermatozoa for 24 hours in BotuFIV® commercial SOF-FIV (BotuPharma©), being randomly assigned into the following experimental groups: Control Group (without adding FF); FF 5% Group (supplemented with 5% FF) and FF 10% Group (supplemented with 10% FF). Following fertilization, the presumed zygotes were transferred to BotuFIV® commercial CIV medium (BotuPharma©, 10% BFS) and cultured *in vitro* for 8 days. Embryo development was evaluated through the cleavage (day 2) and blastocyst formation rates, morphology, and kinetics (day 8). The embryos obtained on the 8th day were used in real-time PCR (three replicates with a pool of five embryos each) in order to analyze the expression of genes related to embryo quality. All results are presented as mean ± standard deviation. Experiments were made with a total of 841 COCs and the statistical analysis was made using the SigmaPlot 14.0 software (Systat Software Inc.). Data were subjected to one-way ANOVA, with Tukey's post-test being used between means that were found to be significantly different ($P<0,05$). There was no significant difference between treatments in relation to cleavage rates, blastocyst formation rates, morphology, and kinetics. About the PCR results, the FF 10% Group presented a significantly lower expression of the IFNT-tau ($P=0,003$) and SOD2 ($P=0,01$) genes when compared to the Control Group, while there was no difference between the Control and 5% FF Groups. Both these genes are related to embryo quality, with IFNT-tau being an essential protein in the maternal recognition of pregnancy and SOD2 being an important protein in the protection of the mitochondria against oxidative stress. Despite these results, the FF 5% Group displayed significantly decreased expression levels of heat shock protein HSP70 ($P<0,001$) and of the pro-apoptotic protein BAX ($P=0,015$), in comparison to the Control Group. These results imply that 5% is the most appropriate concentration for supplementing the IVF medium with FF, generating embryos with reduced expression of genes related to thermal stress and apoptosis.

Animal Reproduction

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

OPU AND IVF

Effect of linoleic acid supplementation during IVM on lipid content and nuclear maturation of cat oocytes

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Aiming at the conservation of endangered felids, the formation of cryobanks is crucial. In this sense, the domestic cat is an important experimental model for developing and applying strategies to improve cryopreservation and associated reproductive biotechnologies in these endangered species. The COC viability after cryopreservation is substantially influenced by the intracellular lipid content, which although presents important cellular functions, is strongly increased during IVM. The aim of this study was to investigate the effect of linoleic acid (LA), as a lipid modulator, during IVM on the total lipid content and the nuclear maturation rate of cat oocytes. After elective sterilizations, oocytes were recovered from ovaries by slicing technique, and COCs were selected based on the presence of an intact and homogeneous cytoplasm and, at least, two layers of cumulus cells. Then, COCs were allocated into two groups for IVM: Control (CONT) (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/LH) or LA (same IVM medium as CONT, but supplemented with 100 µM of LA) for 28 h at 38.5 °C, in 5% O2, 5% CO2 and 90% N2. The experiment was divided into two steps: the first one assessed the lipid content evaluation, and the second the nuclear maturation (MII) rates. After IVM, COCs from each experimental group were denuded with hyaluronidase, fixed in 4% paraformaldehyde for 40 min, and stored in phosphate-buffered saline at 4 °C. The lipid content was analyzed by oocyte staining with Oil Red O solution (Sigma Chemical Co.). Images of each structure (CONT, n=16; LA, n=23) were captured and evaluated for the stained area fraction per oocyte total area using Image J software. Results from lipid content were analyzed by unpaired Student's t-test. For nuclear maturation assessment, four IVM replicates were performed (CONT, n=120; LA, n=126). For reaching that, fixed oocytes were stained with HOECHST 33342 (Sigma Chemical Co.) to evaluate the nuclear configuration under a fluorescence microscope. The results showed that the LA reduces ($P<0.05$) the lipid content of feline oocytes compared to the CONT group. However, no differences ($P>0.05$) were observed in nuclear maturation (MII) rates between CONT (59.2%) and LA (47.6%). In conclusion, LA supplementation during IVM reduces intracellular lipid accumulation, although it does not affect the efficiency of IVM in cat oocytes.

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