Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Shearing ewes at mid-pregnancy induces changes in uterine artery blood flow and placentome size

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Placental growth in sheep occurs mainly from day 30 to day 90 of gestation, but beyond day 90, placenta function varies. Shearing ewes at mid-pregnancy impacts placental and fetal growth. Therefore, it is widely used by farmers breeding sheep under extensive conditions, as natural grassland availability is insufficient to fulfil the nutritional requirements of pregnant ewes. The aim of this study was to compare the uterine and placentome blood flow and the placentome size in ewes that were sheared or not during the second third of gestation. The study was conducted at the Campo Experimental N°1 of the Facultad de Veterinaria, Migues, Uruguay (34° S), with 24 Australian Merino multiparous ewes. All ewes remained grazing natural grasslands under extensive conditions, with free access to water. During the natural breeding season (March-April), estrus was synchronized with two i.m. administration of 160 µg/dose of PGF2α analogue (Delprostenate, Glandinex, Universal Lab., Montevideo, Uruguay), 7 days apart. After the second PGF2α treatment, ewes were exposed to vasectomized marking rams, and marked ewes were inseminated with fresh semen. Pregnancy was determined by transrectal ultrasonography 45 days after artificial insemination, and only pregnant ewes carrying a single fetus were included in the study. Fourteen days before shearing, pregnant ewes were equally and randomly assigned to two groups of 12 ewes each: 1) ewes were sheared on day 90 (range: 88 to 92; group SHE), or 2) ewes remained unshorn (group CONE). SHE ewes were sheared on the same day in July (winter) with the Tally-Hi procedure, beginning at 8:00 am. The non-sheared ewes were handled similarly, being moved to the pen close to the shearing shed. Shearing was performed using a high comb (Heiniger Blizzard, Swiss), leaving a wool remnant of 8 mm. The placentome and the uterine arteries' blood flow and the placentome size were recorded 14 days before shearing, and 6 and 26 days after. The ultrasound examination was performed by the same experienced and trained operator in a quiet and dimly lit room, with the animals restrained in a standing position, using a MyLab One Vet ultrasonic device equipment (Esaote, Genova, Italy) with a multifrequential linear probe (8 MHz) coupled to a PVC tube extension probe. One placentome was randomly identified by B-mode, and the image was saved to measure its area using imageJ™ software (US National Institutes of Health, Bethesda, Maryland, USA). Therefore, the blood flow velocities and indices were recorded. The uterine and placentomes arteries were identified using color Doppler ultrasound and evaluated using the pulsed wave Doppler. The calliper was placed in the central portion of the vessel to determine the peak systolic velocity (PSV), the end-diastolic velocity (EDV) and the resistance index (RI=[PSV-EDV]/PSV). Data were analyzed with a mixed model (SAS on Demand for Academics), including in the model, the treatments (SHE vs CONE), the time before and after shearing as repeated data, and the interaction between treatments and time. As it could not be determined which was the same placentome on each measurement, data from placentome blood flow and size were compared between groups independently at each time point using a mixed model, including treatment as a fixed effect and placentome area as a covariate. The PSV was not affected by shearing, but it significantly increased on days 6 and 26 after shearing ($P<0.00001$, for both comparisons). There was a tendency to an effect of shearing ($P=0.1$) and an interaction between treatments and time ($P=0.1$) on EDV of the uterine artery. There was also an effect of time ($P<0.0001$), as EDV increased significantly 6 days and 26 days after shearing ($P<0.0002$ and $P<0.0001$ respectively). The RI only varied with time ($P=0.0004$), decreasing from 6 to 26 days after shearing ($P=0.003$). Shearing PSV and EDV significantly decreased placentome blood flow (40.1 ± 3.0 cm/s vs 30.0 ± 3.7 cm/s, $P= 0.051$, and 17.7 ± 1.6 cm/s vs 11.2 ± 1.6 cm/s, $P= 0.008$, respectively) on Day 26. There was no treatment effect on blood flow velocities and RI on the other days. The area of the placentomes was greater in SHE than CONE ewes on Day 26 (6.5 ± 0.5 cm2 vs 5.0 ± 0.4 cm2 respectively, $P=0.035$). In conclusion, shearing ewes during the second third of pregnancy triggered an increase in the placentome size and some changes in its' blood flow, which might partially explain the changes that are observed by shearing on the fetal-placental unit.

Keywords: gestation; ovine; placentome vascular indices; fetal-placental unit

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FEMALE REPRODUCTIVE BIOLOGY

Influence of feeding type on neonatal clinical and body development in dogs

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During the neonatal period, dogs are highly susceptible to adverse conditions due to their immaturity and underdevelopment compared to other domestic species. Therefore, meticulous care is crucial for neonatal survival, including feeding management, especially for partially or fully orphaned, sick, and weak neonates. In these cases, artificial feeding becomes essential; however, the effects of different nutritional management modalities on puppy development during the neonatal period are not well understood. Thus, the aim of this study is to evaluate the impacts of feeding with milk replacer for dogs on the potential for body and clinical development during the first 30 days of canine neonatal life. Experiment Approval: CEUA-FMVZ-USP 2639060922. Subjects: Thirty-one Golden Retriever puppies, born from young and clinically healthy female dogs (n=4), submitted to elective cesarean section at term. Procedure: Immediately after birth, the neonates underwent routine stimulation in the delivery room and remained under colostrum breastfeeding for 24 hours with their respective mothers. After this period, the neonates were allocated into two feeding groups: the maternal group (Control - puppies kept on maternal breastfeeding; n=7) and the artificial group (puppies fed solely with commercial milk replacer formula for dogs; n=24). The puppies were breastfed at intervals of 2 hours (1st to 3rd day), 3 hours (4th to 9th day), 4 hours (10th to 15th day), 5 hours (16th to 22nd day), and 6 hours until weaning. To monitor adequate milk (maternal or milk replacer) intake, puppies were weighed before and after each feeding session. Daily clinical evaluations were conducted, including body temperature measurement, feces consistency analysis by score (0-13, with 0 indicating liquid feces and 13 indicating hardened feces), neurological examination for the presence of positive thermotropism reflex, nose stimulation reflex, sucking reflex, ano-genital reflex, pain withdrawal reflex, magna reflex, flexor tone (up to the 7th day of life), and extensor tone (from the 8th day to the 30th day). Body development analysis included weight assessment (in grams) of each puppy through linear regression, considering the periods of 0-10 days and 11-30 days. Weekly blood glucose measurements were conducted until the 30th day of life, totaling 4 evaluations per puppy. Data were analyzed using repeated measures over time, with a significance level of 5%. Body reflexes were analyzed descriptively. Results: For feces consistency evaluation, the maternal group exhibited a lower score (4.5 ± 0.2) compared to the artificial group (6.5 ± 0.03), regardless of the evaluation time point. The maternal group exhibited a higher weight gain during the first and second periods compared to the artificial group. However, there was no difference in the time elapsed for each group to reach half of the weaning weight (16.0 ± 0.4 and 14.3 ± 1.2 days for the artificial and maternal groups, respectively). There was no difference between the groups for body temperature and blood glucose levels; however, an increase in temperature and a decrease in blood glucose were observed over time, regardless of the experimental group. Regarding the analysis of neurological reflexes such as nose stimulation, suction, response to pain, flexor tone, and extensor tone, the developmental pattern was similar between the groups, maintaining adequate scores until weaning. A reduction in the magnus reflex was observed from the second week of life, similarly between the groups. Temporally, there was a reduction in the positive thermotropism reflex and the ano-genital reflex from the second week, especially in the maternal feeding group, followed by a more pronounced decrease in the fourth week, demonstrating progressive neonatal neurological maturation as the puppy grows and develops. In conclusion, neonatal clinical performance is not affected by artificial feeding, although weight gain during the period is greater in puppies kept under maternal nursing, suggesting that the nutritional composition of commercial substitutes is still inferior to maternal milk. Furthermore, feeding with commercial formulas alters stool consistency, tending towards fecal dryness.

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FEMALE REPRODUCTIVE BIOLOGY

Hematocrit, total plasma protein and total serum calcium values in pregnant and lactating female canine

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In the canine species, pregnancy and lactation consist of physiological periods composed of several metabolic changes resulting from hormonal changes and the increase in energy demand of the organism as a whole, which mainly requires more careful and judicious nutritional management (1). Therefore, the present study aimed to analyze the values of hematocrit, total plasma protein and total serum calcium in pregnant and lactating female canine. This project was approved by the Unicentro Animal Ethics Committee (protocol 021/2022). Eighteen bitches were evaluated, aged between two and six years, weighing between four and 15 kg, which were divided into four experimental times, being: time 1 (T1; n= 9) animals less than 45 days of gestation, which had their pregnancy monitored and subsequently moved to T2 (n= 7), consisting of samples from female dogs with more than 45 days of gestation; T3 (n= 12) composed of samples from lactating bitches, 15 days postpartum, which had their lactation monitored and moved to T4 (n= 12), consisting of samples from females 45 days postpartum. Were collected 2 mL of blood through cephalic or jugular venipuncture for analysis of hematocrit using the microhematocrit technique, total plasma protein with the refractometer and total serum calcium with a semi-manual biochemical device calibrated for use of the commercial Calcium Arsenazo Liquiform Kit. The hematocrit, total plasma protein and total serum calcium values obtained were all tabulated and subjected to analysis of variance (ANOVA), followed by the Tukey test, with a significance level of 5%, using the Instat Graphpad statistical software. The hematocrit values in T1 were 37.22±4.38b, in T2 37.28±4.07b, in T3 36.50± 3.75b and in T4 43.25±4.88a, being significant difference was detected (p=0.001). Samples from animals collected at T1 (less than 45 days of gestation), T2 (more than 45 days of gestation) and T4 presented values within normal parameters for the species, while at T3 (15 days of lactation) they were below the standard (2). However, the results of T1 and T2 were at the lower limit. The mean total plasma protein at T1 was 6.68±0.88a, at T2 it was 6.62±0.94a, at T3 6.64±0.97a and at T4 7.22±0.76a, no statistical difference was observed (p=0.065). All values were within the standard for the species (3). In relation to total serum calcium, the mean values were 9.38±1.24a at T1, 9.14±0.79a at T2, 8.90±1.26a at T3 and 8,72±1,27ª at T4, no statistical difference was observed (p=0.625). It was possible to observe that in T3 and T4, which correspond to samples from bitches in the lactation period, the average calcium values were lower than the reference values (2). However, no female dog showed signs of hypocalcemia during the peripartum or lactation period, calcium supplementation was not necessary.

Keywords: anemia; canine; lactation; pregnancy.

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FEMALE REPRODUCTIVE BIOLOGY

Effect of epidermal growth factor concentrations as a signal for the expansion of cumulus cells and *in vitro* maturation of Spix's yellow-toothed cavy (*Galea spixii* Wagler, 1831) oocytes

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Epidermal growth factor (EGF) constitutes a family of polypeptide proteins that signal, through cellular receptors and gap junctions, the genetic activation of mural cells, the expansion of cumulus cells and assistance in events involving oocyte meiosis. In this sense, the use of EGF during *in vitro* maturation (IVM) of oocytes has been a fundamental approach in understanding the reproductive mechanisms of different wild rodents. In Spix's yellow-toothed cavy, a wild rodent adapted to the Caatinga biome, this possibility offers an opportunity to establish protocols involving the oocyte manipulation, to understand reproductive mechanisms related to embryo production, and to genetic conservation of the species. Therefore, we aimed to evaluate the effect of two concentrations of EGF (10 vs. 50 ng/mL) on the expansion of cumulus cells and nuclear maturation of Spix's yellow-toothed cavy oocytes. All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 23091.010566/2017-20) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 60428-1). Then, seven Spix's yellow-toothed cavy were used for ovarian recovery. The cumulus-oocyte complexes (COCs) were recovered by slicing, classified under stereomicroscope and only oocytes with more than one layer of cumulus cells and homogeneous cytoplasm were used for IVM. The COCs were transferred to the IVM medium composed by TCM-199 with 0.2 mM sodium pyruvate, 10% fetal bovine serum, 100 µM cysteamine, 10 µg/mL FSH/LH, 1% antibiotic/antimycotic solution, and 10 ng/mL (EGF10 group) or 50 ng/mL (EGF50 group). Oocytes were divided randomly in both groups and matured *in vitro* for 24 h at 38.5 °C in a humidified atmosphere with 6.5% CO2. Immediately after the IVM, oocytes were evaluated for the degree of cumulus cell expansion under a stereomicroscope, where they were classified as: score I (no expansion), score II (minimal expansion), score III (intermediate expansion) and score IV (maximum expansion). Moreover, denuded oocytes were stained with the Hoescht 33342 probe to visualize their nuclear stage under a fluorescence microscope, where those in metaphase II were considered matured. All results were expressed as mean ± standard error and means were compared using Fisher exact test with $P < 0.05$. Thus, after three repetitions (four-six ovaries per repetition), 106 viable immature oocytes were recovered, resulting in 7.6 oocytes/ovary, which were randomly distributed between EGF10 and EGF50. No difference ($P > 0.05$) was observed between EGF10 (score I: 1.8% ± 1.5 [1/54]; score II: 13.0% ± 1.2 [7/54]; score III 29.6% ± 4.2 [16/54]; score IV: 55.6% ± 2.6 [30/54]) and EGF50 (score I: 9.6% ± 0.3 [5/52]; score II: 11.5% ± 3.5 [6/52]; score III 34.6% ± 9.0 [18/52]; score IV: 44.2% ± 7.5 [23/52] in terms of cumulus cell expansion. This same characteristic was repeated after analyzing the nuclear stage (EGF10: 63.0% ± 2.9 [34/54]; EGF50: 51.9% ± 1.5 [27/52], $P > 0.05$). In conclusion, in terms of expansion of cumulus cells and *in vitro* maturation observed in the Spix's yellow-toothed cavy's COCs, both evaluated concentrations of EGF ensures rates maturation rates greater than 50%. These results represent the first steps towards understanding oocyte maturation in Spix's yellow-toothed cavies.

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FEMALE REPRODUCTIVE BIOLOGY

Effect of *Citrus sinensis* essential oil on cytoplasmic maturation and mitochondrial pattern of porcine oocytes

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The development of oocyte competence depends on the biochemical and structural transformations that occur in its cytoplasm during oocyte maturation. One of the main events described in cytoplasmic maturation is the remodeling and migration of mitochondria. It's wisely that mitochondria play a dynamic role in the metabolism and redox homeostasis of oocytes, and the redistribution of these organelles is important to guarantee adequate energy supply to the cell (1). In view of this, the use of substances that favor the migration process of mitochondria in *in vitro* maturation (IVM) is desired given the need to improve protocols in swine. Citrus sinensis essential oil (EOCS) is a candidate for this purpose, since the use of this natural substance in bovine oocytes increased the quality of embryos (2). Therefore, we evaluated the effect of adding OECS, at concentrations of 10, 30 and 50 µg/mL, to the IVM medium on the cytoplasmic maturation and mitochondrial pattern of porcine oocytes. EOCS was extracted by hydrodistillation of the peels using a Clevenger-type apparatus. The chemical composition was performed using a gas chromatography coupled to mass spectroscopy (D-limonene, 48.5%, α-terpineol, 40.2%, and other compounds, 11.3%. Then, cumulus-oocyte complexes (COCs) were recovered through follicular aspiration of ovaries from slaughterhouses. The COCs were selected based on the morphology of the cumulus cell layer and cytoplasm. Then, COCs were washed and placed in drops of IVM medium, where they remained for 44 h, at 38.5 ºC and 6.5% CO2. The IVM medium was composed of TCM-199 with 0.3 mM sodium pyruvate, 5 µg/mL myo-inositol, 10% fetal bovine serum, 1% antibiotic/antimycotic solution, 20 µg/mL FSH/LH, 10% porcine follicular fluid and 5 ng/mL epidermal growth factor. Additionally, the IVM medium was added with 100 µM of cysteamine (CYS) and supplemented with 10 (CYS + EOCS10 group), 30 (CYS + EOCS30 group) or 50 µg/mL (CYS + EOCS50 group) of the essential oil. After maturation, oocytes were incubated with a MitoTrackerRed probe for 30 min and subsequently photographed under a fluorescence microscope. From the images obtained, these gametes were classified as immature (mitochondria located on the periphery of the cytoplasm) or mature (mitochondria dispersed in the cytoplasm). The mitochondrial organization pattern was also examined, with the stained oocytes categorized into two distinct patterns: pattern A oocytes have a fine and homogeneous distribution, with small granulations spread throughout the cytoplasm, and pattern B oocytes characterized with heterogeneous clusters, highlighted by large granulations dispersed throughout the cytoplasmic extension, where pattern B is desirable. All data were expressed as mean ± standard error, comparisons were performed using the chi-square test ($P < 0.05$). Thus, after three repetitions, 206 viable immature oocytes used in this experiment. No difference ($P > 0.05$) was observed for cytoplasmic maturation rates between groups [CYS: 74.1% ± 6.0 (40/54), CYS + EOCS10: 83.3% ± 6.3 (45/54), CYS + EOCS30: 76.8% ± 9.9 (43/56), and CYS + EOCS50: 71.4% ± 2.8 (30/42]. In contrast, oocytes from CYS + EOCS50 mostly exhibited pattern B in mitochondrial distribution, when compared to those from the CYS group [85.7% ± 11.1 (36/42) and 63.0% ± 3.0 (34/54), respectively, $P < 0.05$]. The CYS + EOCS10 [72.2% ± 8.5 (39/54)] and CYS + EOCS30 [75.0% ± 6.4 (42/56)] groups were similar to the CYS and CYS + EOCS50 for the same assessment ($P > 0.05$). This is an indication that 50 µg/mL EOCS in IVM promotes improvement in the organization and distribution of mitochondria, which is crucial for the function and energetic efficiency of the oocyte (1). In conclusion, EOCS with CYS acts efficiently in promoting the improvement of cytoplasmic maturation of porcine oocytes. Future investigations should be conducted for clarifying the mechanisms of action of this substance on gametes.

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FEMALE REPRODUCTIVE BIOLOGY

Time exposure and cytotoxic evaluation of roscovitine: use of fibroblasts as a model for red-rumped agouti's (*Dasyprocta leporina* Linnaeus, 1758) oocytes

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Oocyte maturation is a biological event that involves changes at both cytoplasmic and nuclear levels. In this sense, the synchrony between these events is of crucial importance for fertilization and embryonic development. Studies conducted in different species have shown that oocyte exposure to meiosis-inhibiting agents, such as roscovitine (RSV), leads to a consequent prolongation of the germinal vesicle stage, resulting in an increase in blastocyst rates (1, 2). Despite this positive effect, oocyte exposure for prolonged periods to this agent can trigger cytotoxic effects (2). Therefore, we used fibroblasts isolated from five red-rumped agoutis as a model to evaluate the cytotoxic effects of 10 µM RSV at different times [12 h (RSV12), 24 h (RSV24) and 48 h (RSV48)]. All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 21/2019) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 71837-1). Then, fibroblasts were previously isolated and stored in a cryobank between the 2nd and 3rd passage (3). After thawing, cell culture was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 2% antibiotic-antimycotic solution at 38.5 °C and 5% CO2. Cells with 60–80% confluence were incubated with RSV for 12, 24 and 48 h. Cells not subjected to RSV were used as a control. All cells were evaluated for morphology, viability with trypan blue, apoptosis levels using acridine orange and ethidium bromide, and cell functionality using analysis of G0/G1 phase by flow cytometry. Data were expressed as mean ± standard error and analyzed using ANOVA followed by the Tukey test ($P < 0.05$). No cellular morphological changes were observed between control and groups cultured with RSV. Moreover, no differences ($P > 0.05$) were verified for viability using trypan blue between control (91.9% ± 0.0) and cultured with RSV (RSV12: 84.7% ± 0.1; RSV24: 70.9% ± 0.0 and RSV48: 81.7% ± 0.1). Regarding to apoptosis levels, a higher percentage of viable cells was observed in all groups (control: 66.3% ± 7.6; RSV12: 65.3% ± 11.4; RSV24: 67.4% ± 5.8 and RSV48: 68.6% ± 6.9) and a low percentage of cells in both apoptosis initial (control: 13.7% ± 2.2; RSV12: 15.4% ± 5.2; RSV24: 17.3% ± 4.7 and RSV48: 12.2% ± 2.7) and late apoptosis (control: 11.4% ± 3.0; RSV12: 14.1% ± 5.1; RSV24: 8.3% ± 2.3 and RSV48: 8.9% ± 3.1), as well as low percentages of necrotic cells (control: 8.6% ± 3.1; RSV12: 5.2% ± 1.8; RSV24: 7.0% ± 2.2 and RSV48: 10.3% ± 2.4). Such aspects can be correlated with low rates of damage at the genetic level, as well as cellular structural damage. Regarding to cellular functionality, cells incubated with RSV presented a greater percentage in the G0/G1 phase (RSV12: 89.9% ± 0.8; RSV24: 74.8% ± 0.8; RSV48: 81.7% ± 0.8) when compared to control (control: 52.2% ± 1.0, $P < 0.05$). In summary, RSV, in the tested concentration and exposure times, did not present cytotoxic effects to fibroblasts derived from red-rumped agoutis and can be suggested for use in inhibiting meiosis in oocytes in this species.

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FEMALE REPRODUCTIVE BIOLOGY

Applicability of transabdominal ultrasonography in the diagnosis of pregnancy in sows

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Ultrasonography is the imaging technology that allows all gestational monitoring in a precise and harmless way, for the female and for the fetus, in addition, from it is possible to detect abnormalities of the pregnancy and fetus, estimate gestational age, evaluate ovaries and uteruses, in addition to verifying fetal viability. In sows, the diagnosis of pregnancy should preferably be made transabdominal, the transducer should be positioned on the ventral abdominal surface, above the last three mammary complexes, from the insertion of the udder to the flank crease. B-mode ultrasound is the best method of early diagnosis, providing high accuracy, but factors such as gestational time, litter size, operator experience, and embryonic losses in the first 35 days of gestation are factors to be considered for the success of the technique (1, 2, 3). Therefore, the present study aimed to evaluate the efficacy of transabdominal ultrasonography in the diagnosis of early pregnancy of sows, in comparison with the observation of non-return to estrus. Six sows, aged between 1 and 3 years, were inseminated with refrigerated semen, with no characteristics of return to estrus detected by the producer. The animals were raised in individual cages in the breeding shed on a small rural property in the city of Guarapuava-PR, Brazil. The students of the study group in animal reproduction of Unicentro, in extension activities, went to the rural property to carry out the diagnosis of pregnancy in sows. For the examination, transabdominal ultrasonography was performed with a Pie Medical Aquila equipment, in B-mode, with a 5 MHz linear transducer, 25 days after artificial insemination. Of the six animals, 66.66% (4/6) were pregnant and 33.34% (2/6) were non-pregnant. In non-pregnant females, the empty uterus presented itself as circular structures, in the cross-section of the uterine horn, or in the form of a band, in the longitudinal section. In pregnant females, it was possible to visualize fluid inside the uterus, representing the embryonic vesicles, in a circular anechoic shape, in addition to the conceptuses, showing the head, thorax, abdomen and limbs, as described in the literature (1, 2). Thus, it can be concluded that ultrasonography demonstrated superiority in determining pregnancy compared to the behavioral evaluation of return to estrus, since, for the farmer, the six sows were pregnant, since no signs of estrus had been detected. As well as the precocity and efficiency of the ultrasound examination in the diagnosis of pregnancy in sows, which increases the reproductive efficiency of the breeding, through the reduction of non-productive days of the sows. Adequate and early diagnosis of pregnancy in pigs is important to adapt the reproductive management of sows, reducing the calving interval and increasing the efficiency of breeding.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Different types of fat-diets associated or not with physical activity do not impact on the histophysiology of females Wistar reproductive system

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It is known that female fertility can be affected by the growing obesity epidemic. One of the factors that cause obesity is the intake of hifh fat-diets and the lack of physical activity. In this scenario, it becomes interesting to evaluate how much each of these factors alone and together can affect the histophysiology of the female reproductive system of Wistar rats. For this, adult female rats were divided (n=8/group) into standard chow group (control, 10% lipid and 20% protein content) without physical exercise (sedentary) - SDCNT; standard chow with physical exercise group - EXCNT; cashew nut group (diet enriched with vegetable fat from cashew nut, 40% lipid and 20% protein content) sedentary - SDCSC; cashew nut group with exercise - EXCSC; lard group (diet enriched with fat of animal origin based on lard, 40% lipid and 20% protein content) sedentary - SDBHP and lard group with physical exercise - EXBHP. During 90 days, the food was offered ad libitum, and consumption was monitored weekly. The physical exercise groups were submitted to 30 minutes of daily physical exercise, following the running protocol on the treadmill (8m/min for 5 minutes, 12m/min for 20 minutes and 8m/min for the last 5 minutes, during the experimental protocol). In the last 15 days, the estrous cycle of the rats was monitored and after this period, in the first estrus, the rats were euthanized for collection, weighing and fixation of the ovaries and uterus, followed by further histopathological analysis. The study was approved by the Ethics Committee of UERN 007/19 and Ethics Committee of UFERSA (PIA10006-2021). The final body weight of the rats revealed a significant increase ($P<0.5$) in the weight of the exercise groups when compared to the sedentary groups, except the SDCSC group compared to the EXCNT group. Furthermore, the feed consumption showed a significant difference ($p<0.05$) between groups SDCSC (14.23+0.83), EXCSC (16.54+0.60), SDBHP (13.69+0.47), EXBHP (16.56+0.58) when compared to both EXCNT (22.15+0.41) and SDCNT (19.19+0.68). The uterine absolute weight of the SDCSC group was also significantly lower compared to the EXBHP group. On the other hand, the weight of the ovaries did not change ($p>0.05$). There was no alterations on ovary and uterus histology ($p>0.05$). The estrous cycle showed that there was a lower percentage ($p<0.05$) of proestrus in the EXCSC group (7.80+2.07) in relation to the SDCNT group (19.94+4.03), but not directly impacted the size and number of estrous cycles when compared between groups ($p>0.05$). Therefore, it can be concluded, based on this experimental model, that both fat-diets and the phisical activity, even thou*-gh they had an impact on weight or feed intake, they did not alter the histophysiology of the female reproductive system. The authors would like to thank the grants to PIBIC (CNPq) and PIVIC UFERSA Scholarship.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Oxidative stress in equine pregnancy: a pilot study of blood enzymatic activity and oxidative damage markers in normal and abnormal cases during the last two weeks prepartum

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Maintaining redox balance is essential for cellular homeostasis, particularly in the orchestration of electron exchange during physiological processes. This balance is sustained by the ongoing formation of reactive species, stemming from aerobic metabolism, which participate in many normal biochemical reactions. However, its accumulation can lead to biomolecule oxidation, compromising the cellular function to varying degrees (1). Pregnancy itself can be considered a challenge to the redox balance, as the pregnant mare is subjected to a great pressure for an increasing energy production. While oxidative stress is linked to complicated pregnancies in human medicine (2), information about the oxidative status during complicated pregnancies in the equine species is currently lacking. This goal of this study is to assess the oxidative status of pregnant mares during the last two weeks before parturition, distinguishing between those with healthy pregnancies and those facing gestational abnormalities. Nine Brazilian Sport Horse crossbred mares, aged 5 to 21 years old and with no prior reproductive history, were enrolled in this prospective cohort study. Pregnancies were categorized as normal or abnormal based on clinical and ultrasonographic parameters. For this, B-mode scanning was performed every 48 hours until parturition for combined thickness of uterus and placenta (CTUP) determination and evaluation of fetal fluids echogenicity (3). Blood samples were collected daily from 320 days of gestation until parturition day by jugular venipuncture. The collection was performed using vacuum tubes internally coated with a clot activator. Immediately after collection, the samples were subjected to centrifugation at 3500 x g for 10 minutes and the blood serum obtained was separated and stored at -20º C until analysis. Enzymatic activity and oxidative damage markers quantification were determined by five assays through UV/Vis spectrophotometry: catalase activity (4); superoxide dismutase activity (5); Ferric Reducing Ability of Plasma (FRAP; 6); malondialdehyde (MDA) quantification (7); and protein carbonyl quantification (8). The data was grouped into week 1(from D-1, with D0 being the day of parturition, to D-7) and week 2 (from D-8 to D-14). Statistical significance was set at $P < 0.05$ for all variables. Five mares exhibited normal gestational parameters throughout the study period and were classified in the normal pregnancy group (NP). Four mares presented with clinical and/or ultrasonographic signs of placentitis or presented with abnormal parturition, including dystocia and premature placental separation, were assigned to the abnormal pregnancy group (AP). Etiological causes for placental diseases presented by the mares in the AP group were not identified. Results revealed that mean values for MDA quantification were higher ($P = 0.048$) in the AP group during week 1, when compared to the NP group for the same time period (89.1 ± 32.86 vs 53.98 ± 19.12 nmol/mL), but other markers showed no significant differences. Malondialdehyde is a byproduct of lipid peroxidation by reactive species, being an important marker of oxidative damage to the cellular membranes (7). This pilot study provides valuable insights into equine oxidative status during the last weeks of pregnancy and calls for further research to establish new potential biomarkers for placental diseases in late-stage equine pregnancies.

Keywords: Enzymatic activity; equine pregnancy; oxidative stress markers

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Morphometric evaluation of Spix's yellow toothed cavy (Galea spixii Wagler, 1831) oocytes after *in vitro* maturation in the presence of epidermal growth factor

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Oocyte growth and ovulation are physiological processes initiated by the increase in luteinizing hormone (LH). Although this hormone is important for the acquisition of meiotic competence in oocytes, this cell does not express LH receptors, and this event is mediated by secondary molecules, such as epidermal growth factor (EGF). In this sense, using EGF during *in vitro* maturation (IVM) of Spix's yellow toothed cavy oocytes, a wild rodent with great ecological and scientific impact, can be a positive approach aimed at understanding mechanisms related to its reproductive physiology, as well as in technological applications, such as *in vitro* embryo production. Therefore, we evaluated morphometrically oocytes derived from Spix's yellow toothed cavy after IVM in the presence of EGF, comparing non-matured and matured oocytes after the IVM. All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 23091.010566/2017-20) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 60428-1). Then, viable oocytes were recovered from fourteen ovaries and matured *in vitro* in TCM-199 supplemented with 0.2 mM sodium pyruvate, 10% fetal bovine serum, 1% antibiotic-antimycotic, 0.1 mM cysteamine, 10 µg/mL of FSH-LH and 50 ng/mL of EGF. All oocytes were cultured for 24 h, 38.5 °C and 6.5% CO2. After IVM, matured oocytes were denuded using 0.1% hyaluronidase, and the structures were identified for the presence and absence of the first polar body (1PB) under a stereomicroscope. Subsequently, photomicrographs were obtained to carry out morphometric analysis using the Image J software, where it was observed the total oocyte diameter (OD), oocyte internal diameter (OID), thickness of the zona pellucida (TZP), ooplasm diameter (OPD), diameter of the perivitelline space (DPS), internal oocyte area (IOA), ooplasm area (OA), perivitelline space area (PSA). All results were expressed as mean ± standard error and means were compared using Fisher exact test with $P < 0.05$. After three repetitions, 52 oocytes were recovered (3.7 oocytes/ovary), of which 29 of these structures presented 1PB. No difference was observed between non-matured and matured oocytes, respectively, according to OD (93.9 ± 0.2 µm and 94.3 ± 1.7 µm), OID (76.7 ± 2.6 µm and 74.9 ± 2.6 µm), TZP (8.9 ± 0.6 µm and 8.5 ± 0.9 µm), DPS (7.7 ± 2.7 and 10.1 ± 2.7 µm), IOA (14564.9 ± 1021.4 µm2 and 13877.3 ± 957.6 µm2), OA (11813.3 ± 1101.2 µm2 and 10412.9 ± 901.7 µm2) and PSA (2751.7 ± 941.6 µm2 and 3464.9 ± 926.5 µm2). These results are positive because it has already been demonstrated in other wild species that lower OD and higher DPS may indicate lower oocyte quality after IVM. However, OPD differed between non-matured and matured oocytes (69.0 ± 3.3 and 64.8 ± 2.8 µm), probably as a morphometric modification of the oocyte in response to the matured status. In summary, this is the first study that describes the morphometric parameters of matured and unmatured oocytes from Spix's yellow toothed cavy. These data are important to understand the events involved in the species' IVM.

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FEMALE REPRODUCTIVE BIOLOGY

Comparison of open and closed-methods for the vitrification of preantral follicles enclosed in canine ovarian tissue

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As an attempt to preserve female genetic material derived from valuable species, the development of different vitrification methods for ovarian tissues has been highlighted. For canids, however, studies on this subject remain scarce. At this sense, we aimed to compare different open and closed vitrification systems on the preservation of preantral follicles (PFs) enclosed in canine ovarian tissues. Six pairs of ovaries from adult females were collected after elective ovariohysterectomy, washed in 0.9% sodium chloride solution, fragmented (3x3x1 mm³) and exposed to closed-vitrification systems as ovarian tissue cryosystem (OTC) and cryotube vitrification (VC), or open methods as needle immersion vitrification (VIA) and solid surface vitrification (SSV). For all methods, fragments were exposed to an equilibrium solution (SE) followed by a vitrification solution (SV) for 10 min each, and finally stored in liquid nitrogen (LN2). For OTC, the fragments were placed inside the device and exposed to SE and SV, then the device was closed and stored. In VC, fragments were exposed to SE and SV and transferred to cryovials and stored. In VIA, the fragments were transfixed with a 30 G needle and exposed to SE and SV, stored in cryotubes, and stored. In SSV, the fragments were exposed to the vitrification protocol and placed in a metal cube in contact with LN2, transferred to cryotubes and stored. After a week, the samples were warmed, and the non-vitrified (control) and vitrified fragments were evaluated for PFs morphology, morphometry, viability, and ultrastructure by scanning electron microscope (SEM). Data were expressed as mean and standard error and compared by ANOVA followed by Tukey test or PLSD Fisher test ($P<0.05$). For PFs morphology, best results were achieved using the VIA system that provided 71.8 ± 9.4% morphologically normal PFs, similar as observed for fresh control group, 65.3 ± 7.67% ($P<0.05$). Regarding PFs morphometry, there were no difference among treatments regarding primary follicle diameter, but only OTC (21.3 ± 0.4 µm), VIA (19.2 ± 0.83 µm) and VC (19.8 ± 0. 61 µm) maintained oocyte diameter similar ($P > 0.05$) to fresh control (20.6 ± 0.5 µm). The same was observed for the nucleus diameter, in which the control group 9.8 ± 0.2 µm only differed ($P<0.05$) from SSV group (8.47 ± 0.25 µm). The other diameters for primary and secondary follicles did not differ between the vitrified groups and the fresh control ($P>0.05$). For viability, only VIA maintained follicular viability (80.6 ± 4.6%) similar to fresh control group (88.9 ± 3.1%) ($P<0.05$), while other treatments varied from 70% to 74%. Through SEM, we detected damage to the ovarian surface caused by vitrification in the organization of stromal cells, being the stromal tissue disorganized mainly in OTC and VIA treatments when compared to the control group. VC and VSS were able to maintain tissue organization with a reduction in cells compared to the fresh group. In conclusion, despite some ultrastructural damage was detected at the use of VIA and OTC, these were the most effective methods to preserve morphological characteristics and viability of PFs enclosed in canine ovarian tissues.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Effects of the addition of colony-stimulating factor 2 to the culture medium with or without serum on early embryo development and postnatal outcomes: preliminary results

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Colony-stimulating factor 2 (CSF2) is a cytokine secreted by the pregnant uterus during early gestation in several species, including cattle. This cytokine is likely to play a role in programming early embryo development and cause effects on fetal development (1) with reflects on postnatal features of the resultant calf (2). Our objective was to investigate whether supplementation with CSF2 during days 5 to 7 of *in vitro* culture, with or without serum, would affect early embryo development, gestation length, and neonatal features of calves. For *in vitro* embryo production (IVEP), cumulus-oocyte complexes (COCs) were collected from slaughterhouse ovaries (majority Nelore females) and submitted to *in vitro* maturation and fertilization using standard procedures of a commercial laboratory (Apoyar Biotech, Alta Floresta, MT). Matured COCs were fertilized using Y-sorted sperm from a single Nelore sire. Embryo culture followed standard procedures in a humidified atmosphere of 5% CO2 and 5.5% O2, except that half of the putative zygotes were cultured in the presence of fetal bovine serum (FBS) whereas the other half in the absence of FBS. On Day 5 of culture, zygotes were randomly allocated to receive CSF2 or vehicle, forming four treatment groups: Control (0% FBS, vehicle); Control-CSF2 (0% FBS, 10 ng/mL CSF2); FBS (3% FBS, vehicle); and FBS-CSF2 (3% FBS, 10 ng/mL CSF2). We used a yeast-derived recombinant bovine CSF2 (Kingfisher Biotech, Inc., Saint Paul, MN, USA). Recipients (n=132) were synchronized for timed embryo transfer (ET) with an estradiol-progesterone based protocol and, on day 7 after arbitrary estrus, eligible females (presence of a >2 cm² CL) received an IVEP embryo from one of the four treatment groups, which were randomly assigned to each eligible recipient for ET on day 7. Pregnancy diagnosis was performed by ultrasonography on Day 26 post-ET and the presence of an embryo with a heartbeat was considered 'pregnancy'. Embryo crown-rump length (CRLd33) was recorded during this exam. For male calves born (n=31), we recorded birth weight, umbilical cord diameter, and gestation length. Data from 3 female calves (8.8% of calves born) were not included in this study. Statistical analysis was performed using the Proc GLM of SAS to examine the effects of treatment on CRLd33, gestation length, birth weight and umbilical cord diameter. Chi-square test was used to determine differences in pregnancy rate among treatment groups. A P-value of <0.05 determined statistical significance and a P-value <0.10 was considered as a trend towards significance. Data are presented as least-square means±SEM. On day 7, 86.3% (114 of 132) of the recipients had a CL and were considered eligible for ET. Pregnancy rates on day 33 did not differ among treatment groups (37.0; 30.8; 44.9; and 33.3% for Control, Control-CSF2, FBS, and FBS-CSF2, respectively; P=0.23). We observed no effect of treatment on CRLd33 (1.09±0.06, 1.08±0.06; 1.04±0.05; 1.08±0.06; for Control, Control-CSF2, FBS, and FBS-CSF2, respectively; P=0.92), nor on gestation length (298.2±2.7, 300.0±2.3, 297.4±1.8, 302.0±2.2, respectively; P=0.43), birth weight (45.5±2.9, 41.1±2.4, 40.7±1.9, 45.9±2.3, respectively; P=0.25), or umbilical cord diameter (1.36±0.2, 1.66±0.2, 1.61±0.1, 1.77±0.2, respectively; P=0.54). Contrasts among groups containing FBS or not (FBS and FBS-CSF2 vs Control and Control-CSF2) and CSF2 or not (Control-CSF and FBS-CSF2 vs Control and FBS) did not detect any effect on the studied outcome variables. Finally, a trend (P=0.09) was observed for a greater birth weight in FBS-CSF2 compared with FBS (45.8±2.3 vs 40.7±1.9, respectively). In conclusion, addition of CSF2 to *in vitro* culture from days 5 to 7 of development did not affect pregnancy-related outcomes and neonatal characteristics of Nelore male calves, except for a tendency for a greater birth weight if CSF2 was added to a serum-containing medium.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Evaluation of different doses of aripiprazole exposure on the reproductive system of female Wistar rats

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It is known that Aripiprazole is a potent third-generation antipsychotic and antidepressant used in various psychiatric treatments. However, as it acts directly on serotonergic, dopaminergic and noradrenergic receptors, the question arises as to whether there are possible impacts of this drug on the female reproductive system. Since our laboratory has been working with the effects of aripiprazole on the male reproductive system, an initial study on the possible effects of subchronic aripiprazole exposure on the cyclicity and histology of female gonads was performed. This study was approved by the Ethics Committee of UERN 006/21 and the Ethics Committee of UFERSA 23091.014948/2019-20. Adult female Wistar rats (n=6/group) from control group (CTRL), group treated with 0.3mg/kg (EXP1), 3.0mg/kg (EXP2) 6.0mg/kg (EXP3) of aripiprazole were treated during 15 days and the estrous cycle of the rats was monitored. The control group received vehicle solution in the 1ml/kg of volume (Dimethylsulfoxide – DMSO + saline solution) and the treated groups received aripiprazole diluted in vehicle solution in the same volume. The treatment was performed by gavage. After the end of the treatment, in the first estrus, the female rats from each group were weighted and euthanized by saturation anesthetic (xylazine and ketamine) and laparotomy was performed. Vital organs (kidney, adrenal, heart, liver, brain, pituitary and thyroid) were collected and weighed. The ovaries and uterus were collected, weighed and fixed in modified Davidson's solution (MDF) to histopathological evaluation. Statistical analysis was performed using ANOVA and Tukey test for parametric data and Kruskal Wallis and Dunn's test for non-parametric data ($P<0.05$). No alterations were observed in the final body, absolute and relative weights of reproductive and vital organs of the rats exposed to different doses of aripiprazole when compared to the CTRL group. However, when compared between the experimental groups, the relative weight of the pituitary was significantly increased in the EXP1 group (0.07+0.01mg/g) in relation to the EXP2 group (0.04+0.01mg/g). The evaluation of the estrous cycle showed a significant decrease in the number of estrus in the EXP3 group (2.4+0.5) when compared to the CTRL group (5.2+0.7). However, no histological changes were observed in the uterus and ovaries sections ($p>0.05$). In addition, the percentage of distribution between primordial, primary, growing and antral folicles and even corpora lutea were similar between the groups ($p>0.05$). Thus, exposure to aripiprazole during 3 ovulatory cycles was not able to impact the female reproductive system. However, more studies with longer periods of treatment are essential to assess the real safety of this antipsychotic therapy on cyclicity and even fertility in females.

Animal Reproduction

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FEMALE REPRODUCTIVE BIOLOGY

Effect of β-caryophyllene on the meiotic resumption status and reactive oxygen species reduction during bovine oocyte *in vitro* maturation

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The *in vivo* follicular environment plays an important role for successful oocyte differentiation, maturation, and subsequent fertilization. Among the important molecules in this process, reactive oxygen species (ROS) stand out because they act as signaling molecules, regulating meiotic resumption (1). However, high levels of ROS can induce chromosomal errors and affect the oocyte's developmental competence. Thus, increased ROS generation *in vivo* is limited by a variety of intracellular antioxidants, which are not observed in the *in vitro* environment during reproductive biotechniques (2). Consequently, it is important to evaluate the addition of an antioxidant substance as a supplement to complex media *in vitro*, to assess its ability to maintain oocyte meiotic resumption and ROS levels in this environment. Therefore, we aimed to evaluate the ability of a natural antioxidant, β-caryophyllene, in preserving metaphase II rates and regulating ROS levels during *in vitro* maturation (IVM) of bovine oocytes. Ovaries were selected from the slaughterhouse and transported to the laboratory in a thermal container, immersed in a saline solution (37 °C; NaCl, 0.9%) and supplemented with 0.05 mg/mL penicillin. Immature oocytes were obtained by follicular aspiration using a syringe and needle. Viable oocytes were selected for IVM in three different groups: without antioxidants (WA, negative control group), 100 µM cysteamine (CYS, positive control group) and 10 µM β-caryophyllene (B10). After 24 h IVM (38.5 °C; 6.5% CO2), oocytes were denuded and evaluated for the presence or absence of the first polar body (1PB) by stereomicroscope. Then, oocytes were fixed in phosphate buffer solution (PBS) with 4% paraformaldehyde for 15 min and labeled with 10 µg/mL Hoechst 33342 for 15 min to evaluate meiosis resumption in metaphase II (MII). Finally, oocytes were incubated at 38.5 °C in 6.5% CO2 with 10 µM H2DCFDA probe for 30 min to evaluate ROS levels. Oocyte images were obtained with fluorescent microscope (40×) and the arbitrary fluorescence units (AFU) were quantified using ImageJ software (3). All data were expressed as mean ± standard error, comparisons were performed using the chi-square test and ROS levels were analyzed by ANOVA followed by Tukey's test. Significance was set as $P < 0.05$. No difference ($P > 0.05$) was observed in MII rates between groups in the presence or absence of antioxidants [WA (90.0% ± 5.3, 54/60), CYS (90.0% ± 7.9, 44/49) and B10 (80.6% ± 12.5, 50/62)]. The same results were observed in oocyte rates with 1PB [WA (84.0% ± 3.2, 205/244), CYS (86.5% ± 6.0, 224/259) and B10 (82.9% ± 4.6, 219/264)] ($P > 0.05$). However, B10 group (0.48 AFU ± 0.18) was similarly efficient to the CYS group (0.37 AFU ± 0.23), managing to reduce ROS levels compared to the WA environment (1.00 AFU ± 0.59) ($P < 0.05$). These results demonstrate that β-caryophyllene was able to maintain high rates of MII, as well as control the expression of ROS levels during bovine IVM. Moreover, the results were at the same level as cysteamine, one of the antioxidants most used substances in IVM processes, highlighting the importance of the presence of at least one efficient antioxidant (4). Therefore, β-caryophyllene becomes a promising option to use in early stages of reproductive techniques, as it can present results similar to cysteamine with a lower concentration. This study is an important advance in establishing better conditions for the gametes *in vitro*, maintaining characteristics similar to the *in vivo* environment.

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Animal Reproduction

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FEMALE REPRODUCTIVE BIOLOGY

PUNICA GRANATUM L. MAINTAINS STROMAL DENSITY IN VITRIFIED BOVINE OVARIAN TISSUE

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Cryopreservation of ovarian tissue is a biotechnique that allows the possibility of preserving female fertility. However, the cryopreservation process can be harmful to a variety of cells, including gametes. In this context, the incorporation of bioproducts into vitrification solutions has been a promising alternative, as these molecules can reduce tissue damage from oxidative stress. Therefore, the present study aimed to evaluate the effect of the ethanolic extract of Punica granatum L. in the vitrification solution of bovine ovarian tissue on the density of stromal cells. The ovarian cortex fragments were subjected to the standard vitrification solution, which was composed of alpha-MEM; 0.25M Sucrose; 10% Dimethylsulfoxide and 10% Fetal Bovine Serum. Furthermore, the standard solution was added of 10, 50 and 100 µg/mL of the ethanolic extract of Punica granatum L. For all vitrification solutions, the exposure time was 5 minutes. Vitrification of ovarian fragments was performed on a solid surface using a metal plate in liquid nitrogen. Then, the fragments were stored in nitrogen for 5 days. At the end of the storage period, all samples were heated. Part of the fragments were fixed in paraformaldehyde for 12 h, and the other part was incubated *in vitro* for 24 h, followed by fixation. After fixation, the samples were subjected to conventional histological processing. To evaluate stromal density, 10% of all sections in the different groups were evaluated using the IMAGE J software. Data were analyzed using normality test (Shapiro-Wilk) followed by analysis of variance (ANOVA-two way) with post Tukey hoc using Graphpad Prisma 9.0 software. As a result, it was possible to observe a reduction in the density of stromal cells in all vitrified groups when compared to the fresh control ($P<0.05$). However, the fragments vitrified at a concentration of 10 µg/mL of Punica granatum L. ethanolic extract showed stromal density significantly higher than the vitrified control ($P<0.05$) after 24 h of incubation. The preservation of ovarian stromal cells is essential, since it provides structural support and maintenance of follicular architecture, which is important for oocyte quality (1). Given these results, it can be suggested that the addition of 10 µg/mL of the ethanolic extract of Punica granatum L. in the vitrification solution maintains the density of the ovarian stroma cells, being an effective alternative in reducing the damage caused by vitrification.

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FEMALE REPRODUCTIVE BIOLOGY

Standardization of an alternative technique for analyzing toxicity in the reproductive system in Wistar rats: comparison with natural mating

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Rodents are widely used as experimental models in several areas of research as they present criteria of similarity, predictability and homology to humans, which validate them for the area of reproductive toxicology. Over the years, there has been an improvement in reproductive techniques and a better understanding of the physiology of reproduction in rodents, with the implementation of *in vitro* fertilization and manipulation of embryos in mice. For rats, in utero artificial insemination (IUA) is used as the gold standard for evaluating sperm quality and toxicity tests. However, it is an invasive method due to the surgical approach. Therefore, the present study compared reproductive indices through standardization and a less invasive insemination technique with the aim of evaluating the impact on the fertility of Wistar rats through comparison with the IUA technique already described and natural mating. For this, twenty nulliparous adult female rats (70 days) were allocated into four experimental groups (n=5/group): Control group (CTRL) that performed natural mating following a standard protocol; in utero artificial insemination (IUAI) group that performed the standard AI technique; group Alternative insemination without seminal vesicle plasma (AI) and group Alternative insemination with seminal vesicle plasma (AIV). In addition, five male rats were used to carry out the procedures. Firstly, natural mating was performed. At the end of natural mating, pregnancy was confirmed, each female was kept in an isolated box for 20 days and then euthanized with saturation anesthetic (xylazine + ketamine) followed by laparotomy and collection of the ovaries and pregnant uterus to determine the fertility parameters. These males used in the natural mating test were placed to recover their sperm reserves and were euthanized, following the same protocol described for females, and the epididymis were removed to obtain the sperm samples that were used for experimental protocols. The cyclicity of the females was assessed by vaginal washing, when proestrus or the beginning of estrus was confirmed, the females in each group were subjected to an insemination procedure. Females were anesthetized with xylazine and ketamine intraperitoneally. For the IUAI group, after trichotomy and exposure of the uterine horns, in utero artificial insemination of 5x106 spermatozoa occurred. Cauterized, relocated to the abdomen, the females were sutured. For the AI and AIV groups, females anesthetized in the same previous protocol were inseminated using a catheter coupled to a syringe, in which the sperm were deposited at the cervix. For the AIV group, a portion of the seminal vesicle fluid was placed in the female's vagina, mimicking the vaginal plug. After 20 days, the same laparotomy procedure previously described was performed and fertility parameters were obtained. Statistical analysis was performed using ANOVA and Tukey test for parametric data and Kruskal Wallis and Dunn's test for non-parametric data ($P<0.05$). This study was approved by the Ethics Committee of UFERSA 11/2023. Our results did not demonstrate the desired efficiency of the proposed alternative techniques. However, we believe that the medium used to dilute the sperm was what compromised the efficiency of the procedures (PBS + 1%BSA), since the males were fertile (CTRL fertile potential = 80%) and did not interfere with pregnancy or fetal development (CTRL losses post-implantation = 5.4%). Furthermore, the standard in utero artificial insemination technique itself, which used the same samples as alternative inseminations, also did not work, as seen in the fertile potential of the three groups IUAI (0%), IA (0%) and IAV (0%) . Therefore, new experiments will be carried out, using HTF medium (a medium normally used in reproduction clinics) with the purpose of evaluating these techniques again. The authors would like to thank PIBIC-CNPq, PIVIC UFERSA Scholarship.

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FEMALE REPRODUCTIVE BIOLOGY

Lagostomus maximus (vizcacha) as an extraordinary animal model for studies in reproductive biology

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Our comprehension of mammalian reproductive biology has predominantly stemmed from investigations conducted on conventional models such as mice, rats, and humans. However, insights gleaned from non-traditional laboratory subjects, including farm and wild animals, strongly indicate the existence of diverse reproductive strategies across species. A more profound understanding of the mechanisms governing reproduction holds promise for enhancing early diagnosis, treatment approaches, and the development of novel strategies to improve fertility and ensure successful reproduction. In this work, we aim to introduce our study model, Lagostomus maximus (Lm), along with a wide array of results. The South American plains vizcacha, Lm, a hystricognath rodent inhabiting the Pampean region of Argentina, exhibits remarkable reproductive features that distinguish it from most mammalian species. In the 1970s, researchers discovered this rodent stands out as a major poly-ovulatory species among mammals, capable of releasing up to 800 oocytes per estral cycle. Despite this extraordinary poly-ovulatory rate, only 8 to 10 oocytes are successfully fertilized and implanted in the uterus, resulting in the gestation to term of only one or two embryos. The high ovulation rate in Lm is attributed to several physiological traits of the ovary, including a convoluted anatomy that increases the ovulatory surface, the small size of ovulatory follicles, and a preferential expression of the anti-apoptotic BCL-2 protein over the pro-apoptotic BAX protein. This imbalance leads to the down-regulation of apoptotic pathways, promoting continuous oocyte production. This strategy of massive ovulation requires a permanent remodeling of the ovarian architecture to maintain the availability of quiescent primordial follicles throughout the individual's reproductive lifespan. We report in our analysis of autophagy (BECN1, LAMP1, and LC3B-I/II) and apoptosis (BCL2 and ACTIVE CASPASE-3) markers which revealed interactive behaviors between both processes, with autophagy promoting survival or cell death depending on the ovarian structure. The unique reproductive features of the vizcacha extend to the development of the female germ line, which occurs without germ cell attrition during fetal life in stark contrast to other mammals. Noteworthy aspects include uninterrupted pre-ovulatory follicle formation during the 155-day-lasting pregnancy and a pseudo-ovulatory process around mid-gestation, resulting in the generation of numerous secondary corpora lutea with oocyte retention. This pseudo-ovulation divides the gestation into two distinct phases, with the second half marked by increased progesterone levels, promoting mammary gland development and supporting the survival of distal embryos. Furthermore, the pseudo-ovulatory event challenges the conventional understanding of the hypothalamic-hypophyseal-ovary (HHO) axis, as it remains active throughout the second half of gestation, contrary to the typical inhibition induced by elevated ovarian steroid levels in most mammals. This unique strategy in Lm involves increased expression of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) around gestational day 100-120, allowing a heightened sensitivity of GnRH neurons to steroid level changes, ultimately triggering ovulation during pregnancy. Interestingly, during pregnancy, the embryo of the vizcacha develops from a bilaminar disc by cavitation, a process distinct from the folding observed in other mammals. While the flat disc morphology of the epiblast aligns with non-rodent mammals such as rabbits, pigs, cows, and humans, it represents an intermediate state between the mammo-typical bilaminar disc and the egg cylinder of muroids in Lm. The extraembryonic ectoderm, homologous to muroids, remains separated from the epiblast by the visceral endoderm, highlighting a unique embryonic development pattern. Moreover, Lm shows a spatiotemporal pattern of primordial germ cell (PGCs)-associated markers divergent from the early PGC restriction model seen in mice. This pattern conforms to alternative models that are based on a pluripotent population in the embryonic axis, where PGCs are specified later during development. This comprehensive examination of embryonic and fetal development in Lm provides valuable insights from an evolutionary and phylogenetic perspective, shedding light on both similarities and differences with other mammals. For all the aforementioned reasons, we propose the plains vizcacha as an exceptional model for investigating mammalian reproductive biology. Finally, although the vizcacha has been described as a massive poly-ovulator with spontaneous ovulation, there is evidence, especially related to penile morphology and oocyte morphology, suggesting that there may be mechanisms of induced ovulation. In the future, our perspective is to deeply study the ovulatory phenomena of the species to determine if it is possible that mechanisms of spontaneous ovulation aimed at eliminating non viable germ mass and mechanisms of induced ovulation aimed at the maturation and release of quality oocytes for reproductive purposes (eu-ovulation) can coexist.

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FEMALE REPRODUCTIVE BIOLOGY

Effect of Pre-service and Pre-lambing Mineral Supplementation on Gestation Rate, Birth and Weaning Weights, and Lamb Survival in Australian Merino Ewes

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Inadequate mineral intake or incorrect proportions affect reproductive function in sheep in the first instance. The objective was to determine the effect of the minerals zinc (Zn), selenium (Se), iodine (I) and cobalt (Co) in different combinations, before service and pre-lambing, on gestation rate, birth and weaning weights, daily gain and lamb mortality in Australian Merino ewes, fed in natural pasture of superficial basalt. These elements besides being of great importance in the reproductive processes in ovines (1), have been detected as marginal in Uruguayan pastures. 346 multiparous ewes were randomly distributed into four groups, with similar numbers of animals according to age, weight, and body condition. Mating was performed at a medium feeding level, and ewes were exposed to 3% rams in April. Fecal samples were taken from 11 animals and analyzed using the McMaster method to determine the parasite load and larval culture to determine the genus. Ewes were supplemented one month prior to mating as follows: Control group (CG): placebos; Group 1 (G1): with Se and Zn; Group 2 (G2): Se, Zn and I and Group 3 (G3): Se, Zn and Co. Minerals were administered in an aqueous solution of Se, I, and Co, and intra-ruminal boluses of Zn. The dose per animal was 5 mg Co, 20 mg Se, 40 mg I, and 80 mg Zn. 35 days after the services was performed transabdominal ultrasonography. 120 days post mating, pregnant ewes were subdivided: GC/0 without supplements, GC/1: placebos; G1/0: without supplements; G1/1: supplemented with Se and Zn; G2/0: without supplements; G2/1: Se, Zn and I; G3/0: without supplements and G3/1: Se, Zn and Co. Sex, birth weight, and weaning weight were recorded. The pregnancy rate was studied using Chi square as well as the survival of lambs at weaning, while birth weight, weaning weight, and daily weight gain were studied using ANOVA ($P<0.05$). The Stata program was used in this study. The parasite load was mild in 10 of the animals (<80 hpg) and moderate in one animal (320 hpg). Larval culture resulted in 100% Haemonchus. A total of 172 lambs and 180 lambs were recorded. There were significant differences in favor of G2 and G3 for pregnant ewes ($P<0.05$). The assumptions to perform a multiple birth frequency analysis were not met; however, we observed more multiple births in the pre-service treated groups (GC: 1, G1: 2, G2: 2, G3: 3). Birth weight, weaning weight, and daily weight gain were not significantly different ($P>0.05$), survival at weaning was significantly different ($P<0.05$) as the proportion of deaths was lower in the groups treated during pregnancy (G1/1, G2/1, G3/1). Fernández Abella (2) have reported that selenium (Se) is effective in improving sperm morphology in rams and fertility in Corridale lambs and Merino ewes. Our results support these findings. Trace elements like cobalt, selenium, iodine, and copper are crucial for fertility, immunity, and fetal development, which are essential for flock profitability (3). Deficiency in these elements can lead to poor reproductive performance, low survival rates, and a higher incidence of birth defects (4). Supplementation of these elements in sheep diets has been shown to increase the number and quality of ovulations (5), which is consistent with our findings. It is concluded that pre-service mineral supplementation (Se, Zn, combined with I or Co) increased the gestation rate and multiple pregnancies, and prepartum supplementation increased lamb survival at weaning.

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X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

EFFECT OF BOVINE OVIDUCT MUCOSA FRAGMENTS POST-VITRIFICATION ON *IN VITRO* FERTILIZATION

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The epithelial cells of the oviduct play a crucial role in various processes of animal reproduction, such as the capture of oocytes, gamete and embryo transport, and the creation of a suitable environment for fertilization and embryonic development. As reported in the literature, *in vitro*, these cells also contribute to creating a more conducive cellular environment for fertilization, thereby enhancing embryo production rates and embryonic quality. With the aim of increasing the efficiency of utilizing these cells during *in vitro* embryo production, the present study sought to evaluate the use of Post-Vitrification Oviduct Mucosa Fragments (OMF) in *in vitro* fertilization. OMFs isolated from uterine tubes collected from slaughtered animals were washed in H 199 medium and vitrified in two steps (3 minutes in a solution of H 199, 20% FBS, 10% DMSO, 10% Ethylene glycol; and after 20 seconds in a solution of H 199, 20% FBS, 20% DMSO, 20% Ethylene glycol, and 0.5M sucrose) and submerged in N2. Subsequently, the OMFs were rewarmed and subjected to sucrose solutions at concentrations of 0.125 and 0.031M and placed in 100µl drops of IVF medium for stabilization. After 18 hours, the fragments were placed along with COCs (Cumulus-Oocyte Complexes) and spermatozoa in the fertilization drop (vitrified FMO group) for approximately 28 hours. After this, the presumptive zygotes followed the laboratory's standard culture protocol. Cleavage and blastocyst formation rates were assessed, along with a qualitative analysis of embryos through the evaluation of the expression of embryonic quality genes. A total of 118 and 126 COCs were used for the Control group (standard IVF) and Vitrified FIV-OMF group, respectively, with no statistically significant difference (P=0.88) observed between the two groups regarding cleavage and blastocyst formation rates. However, gene expression showed significant differences, with higher expression of OCT4 (P=0.031), a crucial player in embryonic development, differentiation regulation, and pluripotency maintenance, and greater expression of IFNT (P=0.001), a key mediator in maternal-fetal communication in ruminants, produced by the embryonic trophoblast and frequently used as a qualitative marker of *in vitro*-produced embryos, in the Control Group. These results suggest the occurrence of events in vitrification that impact OMFs and will influence various aspects of embryonic development, with potential implications for the quality and functionality of embryos produced in the presence of vitrified oviduct mucosa fragments.

Keywords: Oviduct Mucosa Fragments, Vitrification, Embryo

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Immunolocalization of VASA during ovarian development in bovine fetuses

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The VASA protein, also known as Ddx4 (DEAD [Asp-Glu-Ala-Asp] Box polypeptide4) present in invertebrates and vertebrates, is a marker of germline cells in males and females, being fundamental for the differentiation of embryonic stem cells in primordial germ cells. According to the literature, expression in embryos and adults is limited to the primordial germ cells (PGCs) of numerous vertebrate species, such as mice, humans, cattle and others. And in cattle, VASA expression is restricted to the gonad of embryos between 35 and 120 days and analyzes at late stages of fetal development are necessary for further clarification on the expression patterns and their possible functions in gametes. The objective of this work was to evaluate the presence of VASA in the ovaries of bovine embryos and fetuses. Embryos and fetuses (n=12) aged between 1 and 8 months determined by CRL length were collected at a slaughterhouse, and the ovaries were processed for routine histological analysis, 5 µm thick sections were made using a microtome and subjected to the immunohistochemistry technique for VASA (SC-517247) following the manufacturer's instructions. Microscopic analyzes and photomicrographs were performed using an Eclipse Ci-E photomicroscope (Nikon Corporation, Tokyo, Japan) coupled to a NIKON DS-Ri1 digital camera (Nikon Corporation, Tokyo, Japan) and NIS-Elements Basic Research software - NIKON Version 4.0. Immunostaining was observed in the gonad and mesonephron of embryos from 2 to 7 cm CRL (1 month), in all primordial follicles of fetuses between 15 and 98 cm CRL (3 to 8 months) and in antral follicle and oocyte of fetuses between 48 and 62 cm CRL (6 months), concluding that VASA protein is present throughout the ovarian development of bovine fetuses. However, more studies are needed on the function of VASA in folliculogenesis and oogenesis in cattle.

Keywords: VASA, bovine fetus, oogenesis, folliculogenesis

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FEMALE REPRODUCTIVE BIOLOGY

HISTOLOGICAL AND HISTOCHEMICAL ASPECTS OF BOVINE FETUS OVIDUCT

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The oviduct is the site of fertilization and serves to transport the embryo to the uterus. It is comprised of three layers: the serous layer, consisting of the visceral layer of the peritoneum; the muscular layer, composed of smooth muscle; and the mucosa, which consists of epithelium on a layer of connective tissue. The columnar epithelium is of a simple type, with ciliated cells aiding in the transport of gametes and embryos to the uterus, and secretory cells producing oviductal fluid rich in proteins and glycoproteins. These components are known to play nutritional and protective roles in relation to the oocyte and early embryonic development, as well as promoting sperm activation (capacitation). Variations in estrogen and progesterone levels cause changes in the ultrastructural, histochemical, and physiological aspects of this organ. The objective of this study was to analyze the histological and histochemical aspects of the oviduct in bovine fetuses aged between 3 and 9 months, determined by Crown Rump Length (CRL). This analysis was conducted using Hematoxylin and Eosin (H.E) staining and the Periodic Acid Schiff technique (PAS) for the detection of glycoproteins. Oviducts from 16 fetuses were collected at a local slaughterhouse and processed for routine histological analysis. Sections of 5 µm thickness were obtained using a microtome and stained with H.E and PAS. Microscopic analysis and photomicrographs were performed using an Eclipse Ci-E photomicroscope (Nikon Corporation, Tokyo, Japan) equipped with a NIKON DS-Ri1 digital camera and NIS-Elements Basic Research software - NIKON Version 4.0. At 3 months of age, well-developed folds were observed in the serous, muscular, and mucosal layers, with a columnar epithelium containing both ciliated and non-ciliated cells. This pattern persisted across all ages up to 9 months. PAS staining revealed secretion products from the secretory cells at all ages evaluated, indicating that at 3 months, the epithelium already exhibits ciliated and secretory cells, which are actively involved in glycoprotein secretion. This study does not require submission to the CEUA as it utilizes materials collected from slaughterhouses, which are products originating from agricultural activities.

Keywords: oviduct, bovine fetus, histology.

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FEMALE REPRODUCTIVE BIOLOGY

Role of autophagy in the ovary of Lagostomus maximus throughout pregnancy: a rodent exhibiting continuous folliculogenesis and suppressed apoptosis

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Germ cell loss, commonly associated with follicular atresia, has historically been linked to apoptosis, though alternative cell death mechanisms, such as autophagy, play a role. Lagostomus maximus (L.m), commonly called plains viscacha, exhibits reduced or suppressed apoptosis-mediated follicular atresia in both fetal and adult ovaries, maintaining nearly a constant germ cell count from birth to puberty. Our recent findings indicate that autophagy eliminates altered follicles and residual corpora lutea in non-pregnant adult females, creating the space needed to continuously influx primordial follicles into the growing follicular pool, sustaining polyovulation. Aimed to increase our understanding of the possible cooperating role of autophagy and apoptosis in follicular atresia and/or follicular survival we analyzed both programmed cell death mechanisms in a rodent model, the South American plains vizcacha, Lagostomus maximus, whose females show highly suppressed apoptosis-dependent follicular atresia in the adult ovary, with continuous folliculogenesis and a process of pseudo-ovulation during pregnancy. Ovaries from pregnant individuals were collected at early (n=8), mid-term (n=8), and term (n=8) stages of pregnancy. Immunohistochemistry, employing markers such as BECLIN1, LC3BI-II, SQSTM1, LAMP1, and ACTIVE CASPASE-3 (AC3), assessed expression via relative optical density (ROD) and immunoreactive area (IRA). Protein colocalization analysis (LC3B-SQSTM1, LC3B-LAMP1, LC3B-AC3, and BECLIN1-BCL2) was conducted using confocal microscopy, while autophagic vacuole ultrastructure was examined via transmission electron microscopy (TEM). Results, presented as mean ± standard deviation (SD), underwent analysis of variance (ANOVA) with Bonferroni post-tests for multiple comparisons, where $p<0.05$ indicates significance. We report here our analysis of autophagy (BECLIN1, LAMP1, SQSTM1, and LC3B-I/II) and apoptosis markers (BCL2 and ACTIVE CASPASE-3) which revealed interactive behaviors between both processes, where autophagy could promote survival or cell death depending on the ovarian structure. Strong BECLIN1, LC3B-II, SQSTM1, and LAMP1 staining was observed in atretic follicles that also expressed nuclear cleaved-CASPASE-3. Normal follicles showed a slight expression of autophagy proteins but a strong expression of BCL2 and negative expression of ACTIVE CASPASE-3. Transmission electron microscopy revealed a high formation of autophagosomes, autolysosomes, and lysosomes in atretic follicles and a low number of autophagic vesicles in normal follicles. The co-expression of LC3B-SQSTM1, LC3B-LAMP1, and LC3B-AC3 was only detected in atretic follicles, while the co-expression of BECLIN1-BCL2 was only observed in normal follicles. These findings propose a dual role for autophagy in L.m ovaries, actin firstly as a mechanism of cell death synergistically with apoptosis, primarily induced in atretic follicles providing the necessary space for maturation of primordial follicles that continuously enter the growing follicular pool to sustain pseudo-ovulation at mid pregnancy. Secondly, as a main contributor to tissue homeostasis, breaking down and recycling macromolecules that provide essential resources for developing follicles until the completion of pregnancy.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

MEDIUM CONDITIONED BY CUMULUS OOCYTE COMPLEX IMPROVES *IN VITRO* PRODUCTION BOVINE EMBRYOS BY POSSIBLE EFFECT OF HIGH LEVELS OF PROGESTERONE SECRETION

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An important stage of in vitro embryo production (IVEP) is the process in which the presumed zygotes are subjected to successive pipetting to remove the remaining cumulus oophorus (CO) cells, and are then removed from the fertilization drop and transferred to the fertilization drop embryonic cultivation. However, in vivo this loss of communication between the zygote and the CO cells is certainly not so abrupt, demonstrating the need to evaluate the maintenance of this interaction (zygote and CO) for a longer period. The objective of this work was to evaluate the effect of the presence of the mature cumulus oocyte complex (COC) conditioning the in vitro culture medium (IVC) for 48 h. For this, ovaries from a local slaughterhouse were aspirated and COCs classified as grade 1 were selected for both the conditioning of the IVC medium and the IVEP. The maturation, fertilization and embryonic culture processes were carried out according to the laboratory's standard protocol, under conditions of 5% CO2, at 38.5ºC and high humidity. The post-IVM plates had their drops replaced with 100 µL of Synthetic Oviductal Fluid- SOF (10% FBS and 6mg/mL BSA), and then one mature COC was deposited per drop in the COC Group, remaining in the drop until day 2 of culture, when it was removed. After 28 h of fertilization, the presumed zygotes were denuded and randomly distributed between the Control Group (168) and the COC Group (206). Using real-time PCR, we evaluated blastocysts hatched on the eighth day of culture (3 pools of 5 blastocysts each) in relation to the expression of genes involved in physiological processes, such as the octamer transcription factor 4 (OCT4), an important regulator of transcription during early embryonic development and cell differentiation; superoxide dismutase (SOD2) and heat shock protein 70 kD (HSP70) related to cellular protection against oxidative and thermal stress; glucose transporter 1 (GLUT1) involved in the transport of carbohydrates and interferon tau (IFNT), protein produced by the embryonic trophoblast and essential for maternal recognition of pregnancy. Progesterone levels were measured by chemiluminescence in three samples of SOF P4 (0 h), unconditioned medium; SOF P4 (24 h) and SOF P4 (48 h), presence of 1 COC for 24 or 48 h in the IVC drop, respectively. Cleavage rates (day 2), blastocyst formation, developmental kinetics, total cell number (day 8) and embryonic gene expression were subjected to ANOVA (Holm-Sidak post-test, $p<0.05$). There was a statistical difference in the rates of cleavage ($p=0.016$) and blastocyst formation ($p=0.014$) between the COC Group (89.79 ± 4.84; 48.66 ± 6.37) and the Control Group (83.20 ± 5.50 and 38.69 ± 8.98), respectively. There was no difference in kinetic rates and total number of embryonic cells. Analysis of the relative abundance of gene transcripts showed that the COC Group expressed more OCT4 ($p=0.016$), IFNT ($p=0.001$) and HSP70 ($p=0.001$) and less GLUT1 ($p=0.001$) than the Control Group. The mean progesterone secretion levels were 0.1 ± 0.1, 12.2 ± 1.3 and 38.4 ± 15.26 ng/mL in SOF P4 (0 h), SOF P4 (24 h) and SOF P4 (48), respectively. Although embryonic co-culture in the presence of a monolayer of cumulus cells is common in IVEP systems, initial embryonic culture in the presence of COC is unprecedented and appears to allow the continued secretion of factors with beneficial effects on IVC, revealing that the presence of CO is essential not only for the proper maturation and fertilization process, but also for early embryonic development and may be directly related to the high levels of progesterone detected in this experiment. These findings have implications for agriculture as it may contribute to advancements in assisted reproduction programs. Further research is needed to elucidate the specific factors responsible for the observed effects and their mechanisms of action.

Keywords: cumulus oophorus, gene expression, IVEP.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Antioxidant effect of *Citrus sinensis* peel essential oil on *in vitro* environment and nuclear maturation of porcine oocytes

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During nuclear maturation of porcine oocytes, cumulus cells play vital roles in oocyte modulation, and are excellent indicators of the quality of the *in vitro* environment, which is important to ensure success in later stages involving fertilization and development of embryos. Therefore, an adequate *in vitro* environment for the *in vitro* maturation (IVM) of oocytes is a fundamental step towards increasing the rates of *in vitro* embryo production (IVEP). In this sense, the use of antioxidants, such as Citrus sinensis peel essential oil (EOCS) at a concentration of 50 µg/mL, has been reported as a modulator of reactive oxygen species (ROS) of bovine oocytes. Therefore, we evaluated the antioxidant effect of EOCS on the *in vitro* environment and nuclear maturation of porcine oocytes, as a substitute for synthetic compounds. Initially, EOCS was extracted by hydrodistillation of the peels using a Clevenger-type apparatus. The chemical composition of EOCS was performed using a gas chromatography coupled to mass spectroscopy. EOCS presented as constituents D-limonene (48.5%), α-terpineol (40.2%) and other compounds (11.3%). Porcine ovaries were recovered from the slaughterhouse, cumulus-oocyte complexes (COCs) were obtained by follicular aspiration and classified by stereomicroscope as viable according to the number of cumulus cell layers and cytoplasmic homogeneity. Then, oocytes were distributed according to the groups: 100 µM cysteamine (CYS group; synthetic compound) and 50 µg/mL EOCS (EOCS50 group). All oocytes were matured in TCM-199 supplemented with 0.3 mM sodium pyruvate, 5 µg/mL myo-inositol, 10% fetal bovine serum, 1% antibiotic-antimycotic solution, 20 µg/mL FSH/LH, 10% porcine follicular fluid, 5 ng/mL epidermal growth factor, and 100 µM CYS or 50 ng/mL EOCS (44 h, 38.5 °C, 6.5% CO2). After IVM, matured oocytes were analyzed for cumulus cell viability by trypan blue assay, presence of the first polar body (1PB) under stereomicroscope, and number of ROS by H2DCFDA fluorescent probe. For this probe, oocyte images were obtained with fluorescent microscope (40×) and the arbitrary fluorescence units (AFU) were quantified using ImageJ software. All data were expressed as mean ± standard error, comparisons were performed using the chi-square test and ROS levels were analyzed by ANOVA followed by Tukey's test ($P < 0.05$). After three repetitions, 141 viable oocytes were recovered from 54 ovaries (2.6 oocytes/ovary). Regarding cumulus cell viability, EOCS50 [92.4% ± 0.6 (1040/1125)] maintained a higher rate than CYS [88.4% ± 1.1 (604/683)], although there was no difference after the 1PB analysis between the groups [CYS: 74.4% ± 1.6 (32/43); EOCS50: 78.0% ± 1.1 (39/50)]. Furthermore, no difference was observed after analyzing intracellular ROS levels (CYS: 1.00 ± 0.6 AFU; EOCS50: 0.79 ± 0.3 AFU). These results are important because in addition to the EOCS50 did not present toxic effects, it improved the quality of cumulus cells in the *in vitro* environment, being an important factor during porcine IVM, and possibly in the subsequent stages of IVEP. In summary, EOCS at 50 µg/mL showed promise as a modulator of ROS levels during the IVM, demonstrating the substitutive potential for cysteamine in porcine oocytes.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Relationship between subclinical endometritis diagnosis and spontaneous recovery in lactating cows

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The aim of this study was to evaluate the dependence between the time of subclinical endometritis (SE) diagnosis and spontaneous recovery in lactating cows. Postpartum gynecological examinations were performed on 512 Holstein cows, multiparous, from April to September 2022 at a dairy farm (Lat -32°50´02.2´´S, Long -61°41´38.4´´W). After positive diagnosis with endometrial cytology of ≥5 polymorphonuclear neutrophils (PMN), 56 cows were evaluated considering two different times (M) for postpartum evaluation: M1 at 26.7±1.0 d (n=43) and M2 at 48.1±3.5 days (n=13). Each group received a second examination 15 days apart. After a 2° examination, cows were separated into three groups: cows with spontaneous recovery (SR), cows that maintained disease (SE), and cows that developed clinical endometritis (CE). The dependence between the status of uterine health (health or illness) and time of SE diagnosis (M1 and M2) was evaluated with a Chi-square homogeneity test ($P < 0.05$) and t-Student ($P < 0.05$). The t-Student test was used for paired samples. The results showed that 65.1% (28/43) of cows experienced spontaneous recovery, 13.9% (6/43) maintained SE and 20.9% (9/43) developed to CE, at M1. On the other hand, at M2, the disease evolution showed 61.5% (8/13), 15.4% (2/13), and 23.1% (3/13) for SR, SE, and CE groups, respectively. No differences were found between the time of diagnosis and SE evolution. Otherwise, SR cows showed a significant and abrupt decrease of the % PMN at endometrial cytology during the 15 days of the postpartum examination period (12.1±1.7 y 1.0±0.2% PMN, respectively) ($p>0.0001$). In conclusion, our results indicated no dependence between spontaneous recovery and time of subclinical endometritis diagnosis in lactating cows. This study could generate new hypotheses about spontaneous recovery and the time at which some veterinarian practices are made, looking to improve uterine health in Holstein cows.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Impacts of chronic exposure to different high fat-diets and physical activity on the reproductive system of adult *Wistar* rats

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Nutritional imbalances, mainly associated with a high intake of fat-diets, combined with a lack of physical activity, can significantly contribute to the onset of obesity. Furthermore, it is known that the accumulation of fat can directly harm reproduction, promoting hormonal problems as well as fertility issues. To investigate the possible impacts of a chronic exposure to different high fat-diets with or without physical activity on the reproductive system of adult female Wistar rats, these animals were divided (n=8/group) into standard chow group (control, 10% lipid and 20% protein content) without physical exercise (sedentary) - SDCNT; standard chow with physical exercise group - EXCNT; cashew nut group (diet enriched with vegetable fat from cashew nut, 40% lipid and 20% protein content) sedentary - SDCSC; cashew nut group with exercise - EXCSC; lard group (diet enriched with fat of animal origin based on lard, 40% lipid and 20% protein content) sedentary - SDBHP and lard group with physical exercise - EXBHP. The Ethics Committee number of UERN 007/19 and Ethics Committee of UFERSA (PIA10006-2021). The physical exercise groups were submitted to 30 minutes of daily physical exercise, following the running protocol on the treadmill (8m/min for 5 minutes, 12m/min for 20 minutes and 8m/min for the last 5 minutes, during the experimental protocol). The treatment protocol was a chronic exposure (180 days) of these different diets and in the last 15 days, the estrous cycle of the rats was monitored. After that, in the first estrus, the rats were euthanized for collection, weighing and fixation of the ovaries and uterus, followed by further histopathological analysis. Ovaries and uterus were fixed in formaldehyde, processed and stained with eosin-hematoxylin for histopathological analysis. There were no statistical differences observed in the body weight of the animals and also in the uterine and ovarian absolute and relative weights. In addition, no alterations were observed in the number of estrous cycles and in the duration of these cycles between the groups. However, when each phase was analyzed individually, a statistically higher frequency of the diestrus phase was seen in the EXCSC group (54.81+6.41) when compared to the SDBHP group (25.96 + 8.09). In the histopathological evaluation of the ovaries, no significant changes were identified. All groups had well-defined and characteristic gonads. Histopathological analyzes of the uterus showed that both, in the sedentary and in the groups submitted to exercises, a normal histologycal morphology of this organ. Therefore, based on the present experimental model, there was no deleterious effects caused by the different types of high-fat diets and physical activity in the on the female reproductive system. Which, on the one hand, opens new perspectives on the intake of different types of fat on health and fertility studies. The authors would like to thank the grants to PIBIC (CNPq) and PIVIC UFERSA Scholarship.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Preliminary data of SCNT embryo transfer in pigs for xenotransplantation

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The anatomical and physiological resemblances between pigs and humans, coupled with advancements in genetic engineering techniques enabled the creation of genetically modified pigs. This new biotechnology aims to address the increasing need for transplant organs. Consequently, our objective is to establish Brazil's first pig colony specifically for xenotransplantation purposes.

Fetal endothelial cells (E) were used to produce genetically modified clone embryos, which were modified using CRISPR/Cas9 technique and used as nuclear donors for clone embryo production. Three pig genes were knocked-out (TKO) in E cell lines (E-TKO) to reduce the immunological rejection of the xenografts: B4GalN2, CMAH, and GGTA1. Subsequently, oocytes aspirated from pig ovaries obtained in slaughterhouses were used to produce parthenogenetic embryos (PT) or were micromanipulated for somatic cell nuclear transfer (SCNT) to individually reconstruct with the modified E-TKO cells. In Parallel, wild type fibroblast cells (Fb-WT) were also used in SCNT for protocol validation. The embryos were in vitro cultured until the zygote stage (Day 1 or Day 2). The embryos in vitro produced were transferred by laparotomy bilaterally to both oviducts of 14 surrogate sows with synchronized estrous cycles. Other parameters were evaluated during the transfer surgery, such as the type of transfer (oviduct catheterization [OC] or via the fimbriae ostium [FO]) and estrous cycle stage by ovaries observation (pre-ovulatory [PRE] or post-ovulatory [POS]). The surrogate sows were monitored daily, and pregnancy diagnosis was performed by ultrasound exam starting from 30 days after zygote transfer.

Three sows (P1, P2 and P3) received only 60 PT embryos. Two pregnancies were confirmed at 30 days, evidenced by the visualization of vesicular structures and embryonic fluid, but they were reabsorbed between 45-50 days, as expected. P3 did not become pregnant. Two sows (P4 and P5) received 50 E-TKO embryos along with 46 PT embryos, and one sow (P6) received only 96 E-TKO embryos. All these three sows had positive pregnancy at 30 days of gestation; however, the pregnancies did not proceed in any of them due to the non-visualization of fetal bone structures or cardiac movements between 45-60 days of gestation. Subsenquently, P7-P14 received 40, 32, 32, 270, 130, 200, 200 and 210 Fb-WT embryos, respectively. Of these, only P8, P9, and P11 did not become pregnant. Although positive, the evolution of P7, P10, P12-P14 pregnancies after 30 days of gestation did not proceed. P4 and P12 showed, respectively, 2 and 4 embryos in the initial stage of development after necropsy. Regarding the cell type (E-TKO vs. Fb-WT), transfer site (OC vs. FO), and ovulatory stage (PRE vs. POS), E-TKO – OC – PRE sows (n = 3) had a pregnancy rate of 100% compared to 62.5% of Fb-WT – FO – POS sows (n = 8). However, this difference may be more related to the imbalance in the number of sows between groups than to a real difference.

Indeed, most embryonic losses in pigs occur during the first 30 days or at the first third of gestation1. Furthermore, the low efficiency in obtaining cloned piglets through SCNT was already expected. Only 0.5 - 7% of the transferred cloned pig embryos reach full term 2,3. Additionally, the number of embryos transferred was less the ideal, due to limitations in obtaining a greater number of better-quality oocytes and trained personnel for micromanipulation. Another major difficulty inherent to the SCNT technique is finding the appropriate cell line for producing clones. We are currently testing the possibility of cloning with cell lines from different individuals and of different cell types, so that we can proceed with genetic editing. Recent studies recommend transferring a minimum of 150 structures per sow 2, a number that we achieved only in the latest transfers. With the increase in the number of embryos produced and transferred, and with the appropriate cell lines, we hope to achieve the first successful full-term pregnancy of porcine clones.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Evaluation of the toxicity of chromomycin A5, an antitumor bioactive compound, on the *in vitro* development of preantral follicles included in ovine ovarian tissue

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The ovary is the organ of the female reproductive system most susceptible to damage caused by chemotherapy and premature ovarian failure is an important sequel in women undergoing chemotherapy treatment. In this way, investigating natural compounds that are effective against tumor cell lines, and evaluating their toxicity on reproductive function, can provide better alternatives for the development of new drugs in the treatment of cancer. The aim of this study was to assess the toxicity of chromomycin A5 (CA5), a metabolite isolated from marine bacteria of the genus Streptomyces with potent antibacterial and antitumor activity, on the *in vitro* development of sheep preantral ovarian follicles. To this end, fragments of ovarian cortex collected from slaughterhouses were removed and distributed in the following experimental conditions: non-cultured control (NC), *in vitro* culture (IVC) for 1 and 6 days in α-MEM (MEM); α-MEM added with 0.3 µg/mL doxorubicin (DOX) as a positive control; α-MEM added with 100, 200 or 300nM chromomycin A5 (CA5100, CA5200 and CA5300). A total of 2,640 follicles were analyzed for their classification and morphology by classical histology. The means ± SEM obtained from the Control NC (100±0.0) and on D1 MEM (99.2±0.5); DOX (74.3±3.8); CA5100, 200 and 300nM (86.3±4.9; 74.2±5.8; 57.9±4.6, respectively), revealed that the CA5300 treatment showed the lowest follicle survival rate on D1 of culture, when compared to all the other treatments ($P<0.05$). On D6 of IVC, all treatments, DOX (58.3±4.2); CA5100 (61.7±4.5); CA5200 (40.4±5.0) and CA5300 (36.3±2.3), significantly reduced follicle survival when compared to MEM (80.4±8.3). Cell senescence was analyzed by marking lipofuscin granules in the ovarian tissue. The MEM (37.05±0.03) and DOX (105.83±0.26) treatments showed fewer and more senescent cells, respectively, compared to the other treatments. In addition, an analysis of the proliferation antigen (PCNA) was conducted on IVC ovarian tissue for 6 days, where there was a significant reduction in cell proliferation in the groups treated with DOX (145±4.3); CA5100 (133±7.9) and CA5300 (139±2.6), when compared to MEM. The TUNEL test on the ovarian stroma after 6 days of IVC showed a significant increase in DNA fragmentation in the CA5300 treatment (39.7±10.5) compared to the other treatments MEM (9.0±3.1); DOX (23.0±4.9) and CA5100 (24.0±1.0). Exposure of ovine ovarian tissue to 100, 200 and 300nM doses of CA5 after 6 days of IVC resulted in the progressive loss of preantral follicles. The concentrations of CA5 tested in this study suggest toxic effects on the survival of preantral follicles, inhibition of cell proliferation and damage to the ovarian stroma in sheep. However, further analysis is needed to confirm the dose-dependent effect on the death of preantral follicles, by investigating the mechanism of action of chromomycin A5 on the development of ovarian follicles.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Relations between reproductive endocrinology and vaginal cytology of Brazilian tapirs monitored during different environmental seasons in the Cerrado biome

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The Brazilian tapir (Tapirus terrestris) is the largest mammal in South America, being the unique reminiscent representant of the Megafauna in this continent. Despite its immense ecological importance, as it acts as a major seed disperser, its population has been under constant threat due to the advances in agriculture and livestock farming. Unfortunately, a low survival rate of the species has been observed in the Cerrado biome, which is characterized by savannah vegetation associated with high environmental temperatures. Therefore, the study of the reproductive physiology of tapirs is of fundamental importance for the development of strategies for their conservation. The aim was to describe the reproductive endocrine parameters, and correlating them with the vaginal cytology of Brazilian tapirs from the Cerrado biome, monitored at different environmental seasons. After capture and chemical restraint, a total of 24 females were grouped, according to its estimated age, as pre-pubertal (under 16 months), sub-adults (17-45 months), adults (48-180 months) and senile (over 180 months), and their weight was estimated. Blood samples were collected for progesterone and estrogen determination by radioimmunoassay, using a Wizard detector (PerkinElmer do Brasil Ltda) and commercial kits. Moreover, vaginal smears were taken using a sterile swab introduced through the vulvar commissure to the cranial vagina, and stained using the Diff Quick method, according to the manufacturer's instructions. The samples were analyzed under an optical microscope (x400) and the cells were classified as basal, parabasal, intermediate or surface cells (1). Data from female tapirs were also grouped according to the environmental season when collection was conducted during the dry (May to September) and rainy (October to April) seasons from the Cerrado biome. Mean values (± SEM) were compared using the Kruskal Wallis test, followed by Dunns' multiple comparison. A Spearman correlation test was performed to verify the relationship between hormonal concentrations and the proportion of cell types found by vaginal cytology. Females were distributed as 3 prepubertal weighting 85.0 ± 0.0 kg, 5 sub-adults with 169.0 ± 16.0 kg, 15 adults with 220.0 ± 3.39 kg, and 1 senile with 225.0 ± 0.0 kg. There were no differences ($P > 0.05$) in estrogen and progesterone values according to the aging group, whose mean values were 85.1 ± 15.0 pg/mL and 0.2 ± 0.2 ng/mL (prepubertal), 118.3 ± 14.6 pg/mL and 0.1 ± 0.0 ng/mL (subadults), 127.6 ± 16.5 pg/mL and 1.4 ± 0.4 ng/mL (adults), 106.3 ± 0.0 pg/mL and 0.6 ± 0.0 ng/mL (senile), respectively. Regarding vaginal cytology, we identified a predominance of intermediate cells in smears from prepubertal young (67.3 ± 28.6%), subadults (58.2 ± 11.4%), adults (56.7 ± 7.7%) and senile (62.0 ± 0.0%). Moreover, proportions of the cellular pattern showed no difference throughout the cycle and periods analyzed ($P>0.05$). There was a moderate positive correlation between estrogen levels and parabasal cells ($r = 0.4462$; $p = 0.0288$), but no other correlation was found between hormonal concentrations and other vaginal cell types. Additionally, hormonal patterns from female tapirs were well distributed during all the year, and we did not identify any prevalence of estrogen peaks relative to a concentration of estrus occurrence in any of the environmental seasons studied. Based on the data analyzed, it appears that there is no occurrence of reproductive seasonality in the species. Furthermore, vaginal cytology appears to be inefficient in monitoring the endocrine patterns of the species. This is the first description of the reproductive parameters of female Brazilian tapirs from the Cerrado biome and the data obtained provide important information for understanding the reproductive physiology of the species, which can be crucial for the development of strategies for its conservation.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

OVARIAN MORPHOLOGICAL VARIATIONS OF *PSEUDOPALUDICOLA POCOTO* MAGALHÃES, LOEBMANN, KOKUBUM, HADDAD & GARDA, 2014 IN A FRAGMENT OF CAATINGA IN THE NORTHEAST REGION IN A ONE-YEAR CYCLE

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Oogenesis comprises a crucial development process for the perpetuation of most species (1), however, several factors may interfere, extrinsic, such as rainfall, temperature, photoperiod, food availability and breeding sites, and intrinsic, such as hormonal cyclicity and the presence of fat bodies for the production of gametes (2). Little is still known about the reproductive dynamics of neotropical anuran communities, so this study describes the reproductive process of Pseudopaludicola pocoto Magalhães, Loebmann, Kokubum, Haddad & Garda 2014 and the morphological changes that occurred in 13 months resulting from environmental factors existing in a fragment of caatinga. Three mature females were collected per month, for 13 months, in a fragment of caatinga located in the municipality of Passagem, Paraíba, Brazil during the year 2019 and histomorphometric and stereological analyzes were carried out on the gonads of the specimens found. It was seen that rainfall was a common influencing factor in the frequency of all developmental stages, but the influence of temperature occurred only at the most advanced stages. Oocytes found in months of greater rainfall and mild temperatures had greater volume and a rounder shape, with the exception of grade 1 oocytes. High temperatures and dry periods promoted a reduction in volume and oocytes were more susceptible to environmental changes and their frequency. Throughout the cell maturation process, situations that increase the scarcity of nutrients necessary for oocyte development can interfere with their morphology and abundance (3). It was seen that reproductive cyclicity is dependent on abiotic factors on this species, characterized by behavior adapted to the unpredictability of rainfall over the months.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Supplementation of *in vitro* maturation medium with metformin improves the development of bovine *in vitro*-fertilized oocytes toward expanded blastocyst stage

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The rising demands of recruiting *in vitro*-produced embryos for optimizing cattle husbandry encounters some limitations, including the inferior female gamete competence regarding its *in vivo* counterpart. This attributes to degenerative effects mediated by elevated reactive oxygen (ROS) generation in the artificial environment. However, oxidants neutralization attends moderately the expectations, so further antioxidant activity may remarkably enhance oocyte potential, like inhibiting free radical liberation from the respiratory chain as performed by metformin (MT). To test our hypothesis, cumulus-oocyte complexes (COCs) obtained by puncturing the visible antral follicles of abattoir ovaries were selected, basing on the surrounding cells (≥3 compacted layers) along with ooplasm appearance (opaque homogeneous) and randomly distributed in four groups with different concentrations of MT during *in vitro* maturation for 22-24h: MT0=0 (n=139), MT1=0.05 (n=142), MT2=0.1 (n=143) and MT3=0.2 mM (n=151). The *in vitro*-matured COCs were placed in *in vitro* fertilization (IVF) medium containing spermatozoa (concentration= 2X106 per ml) previously separated with mini Percoll gradient; 18 h later the presumptive zygotes were partially denuded and cultured in synthetic oviduct fluid (SOF) + 3% fetal calf serum. Cleavage and blastocyst (BL) rates from six replicates were observed at 48 and 168 h following IVF. Then, 4-5 BLs/group produced from MT0 (n= 18) and MT2 (n= 22) in five independent replications, were submitted to terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay and stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize the apoptotic and viable blastomeres respectively, under a florescent microscope (EVOS M5000 microscope, Invitrogen). The qualitative variances (*in vitro* embryo production results) were analyzed using logistic regression and chi-square. Total cell number and apoptosis index (+TUNEL/blastomeres %) were analyzed by One-Way ANOVA (SAS university software, version9.1); the difference considered significant when P≤0.05. The data are expressed as mean±SEM. MT had no effect (P>0.05) on cleavage (78.8±3.2, 76±3.3, 75.5±2.9, and 76.6±3.6% for MT0, MT1, MT2, and MT3, respectively) and blastocyst (46.4±7.1, 48±4.5, 40.8±2.8, and 44.3±5% for MT0, MT1, MT2, and MT3, respectively) rates. The rate of embryos at blastocyst stage was lower (P<0.05) in MT2 (3.3±1.8%) than in MT0 (14.5±3.4%), MT1 (7.3±2.7%) and MT3 (8.8±3%), whereas rate of embryos at blastocyst expanded stage (27.5±1.8%) was higher (P<0.05) than MT0 (18.1±4.8%) with no difference (P>0.05) among MT0, MT1 (26.5±5%) and MT3 (24.6±14%). The percentage of the embryos at remaining blastocyst stages presented no difference (P>0.05) among groups (early blastocyst: 10.9±2, 12.7±2.8, 9.3±2 and 9.6±1.6%; hatching blastocyst: 1.39±1.3, 0.83±0.8, 0 and 0.72±0.7% or hatched blastocyst: 1.39±1.3, 0.57±0.5, 0.69±0.6 and 0.52±0.5%, for MT0, MT1, MT2 and MT3, respectively). Total cell number (173.77±7) and apoptosis index (19.45±2.12%) in MT2 were higher (P<0.05) than in MT0 (138.33±11.12 and 13.20±1.54%). In conclusion, 0.1 mM metformin added to the *in vitro* maturation medium favors the development of zygotes towards expanded blastocyst stage, although no effect on overall blastocyst production was found. Considering that the blastocysts at expanded stage are usually the preferred category for vitrification, metformin can be useful to generate embryos with the aim of cryopreservation. Nevertheless, the role of metformin on apoptosis in blastocyst requires further investigation. Financial support: CNPq and Fapemig.

Keywords: antioxidants; respiratory chain; expanded blastocyst.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

The uterus contractility activity from *Wistar* rats after *in vitro* aripiprazole exposure

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Aripiprazole is a third-generation drug widely used to treat neurological diseases, mainly schizophrenia and major depression disorder. Due to the lack of in-depth studies on the possible effects of exposure to aripiprazole on reproduction and fertility, our laboratory has carried out a comprehensive study on this topic. As some subtle effects were observed on exposure to different doses of the antipsychotic aripiprazole on the female reproductive system and the analyses of tecidual contractility of reproductive organs had demonstrated being effective to decrease the number of used experimental animals and obtain direct results of the tecidual response to different substances, our objective was to investigate the possible direct effects of this drug on the contractile activity of the uterine horns of adult female rats in estrus phase. All the procedures were authorized by CEEA 007/21 and CEUA 23091.014949/2019-90. For this, two adult female rats were euthanized by CO2 followed by laparotomy for uterus collection. After that, an isolated section of the medium portion uterus horn (3.cm) was incubated in an organ bath to the 15 min of stabilization period under 1g of tension. After that, a 60 mM KCl shock was performed to evaluate the tissue viability. The tissue was washed and submitted to a rest time (10 minutes) and a new 60 mM KCl shock was performed in the same manner. After 30 minutes of the last contraction to KCl, the tissues were incubated with oxytocin and the contractility activity was recorded for 5 minutes, which was considered a control curve. The tissue was washed again and the process was repeated at incubated concentrations of 10 µM, 300 µM and 1000 µM of aripiprazole for 30 minutes and exposed to oxytocin to analyze the contractile profile for 5 minutes. Our data showed no alteration in the Area Under Curve (AUC) in all doses of aripiprazole when compared to the control AUC. However, there was a significant decrease in the frequency of contractions per minute ($p<0.05$) in the 10 µM (0.93+0.17) and 1000 µM (0.60+0.17) of aripiprazole concentration when compared to the 0 µM (control curve, 5.33+0.76). These data suggest a reduction in uterine contractile activity during the estrous phase of the female cycle. This reduction can impact both implantation and the development and/or maintenance of pregnancy. More studies must be carried out to determine what can really happen during the gestational period. The authors would like to thank PIBIC-CNPq, PIVIC UFERSA Scholarship and UFERSA/PROPPG PPP (grant number 23091.014593/2019-02) and PIAP (grant number 23091.005800/2023-42).

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Characterization of vaginal aerobic and microaerophile microbiota and its relationship with the reproductive stage in captive collared peccaries (*Pecari tajacu* Linnaeus, 1758)

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The characterization of the microbiota of the reproductive tract of female peccary could contribute to the knowledge of the reproductive aspects of these animals, enabling progress in the scenario of their conservation. The aim of this study was to characterize the aerobic and microaerophilic vaginal bacterial microbiota of female peccary and evaluate the influence of the reproductive status on this microbiota. To this end, 11 female peccaries at different reproductive status (4 young pubescent, 4 non-pregnant adults and 3 pregnant adults) were physically restrained with a hand net, placed in lateral decubitus, and submitted to blood collection to measure serum progesterone. After cleaning the vulvar region, sterile swabs were used to collect samples from the bottom of the vagina, which were used both for colpocytological analysis and for the identification of microorganisms by culture and bacterial isolation, followed by MALDI-TOF MS analysis. Statistical analysis was carried out using GraphPad Prism® software version 8 for Windows. The results were expressed as mean and standard error (SEM). The normality of the residuals was checked using the Shapiro-Wilk test and the homogeneity of variance was checked using the Bartlett test. The results were considered significant when $p < 0.05$. Serum progesterone levels by reproductive stage were 13.88 ± 6.63 ng/mL for young pubescent females, 10.77 ± 4.98 ng/mL for non-pregnant adult females, and 64.38 ± 10.20 ng /mL for pregnant ones. Regarding the bacterial profile, in general, most microorganisms found belonged to the Firmicutes phylum. In the group of pubescent females, 33.33% of microorganisms from the phylum Proteobacteria were found, with a predominance of the species Alcaligenes faecalis. In the group of non-pregnant adult females, 85.71% of the microorganisms belonged to the Firmicutes phylum, with a predominance of the species Bacillus badius and Staphylococcus microti. In the group of pregnant females, 54.55% of the microorganisms belonged to the Firmicutes phylum, with a predominance of the species Bacillus cereus and Mammaliicoccus sciuri. There was no significant correlation between serum progesterone levels and vaginal cytology. This study is the first scientific characterization of the vaginal microbiota of female peccary and demonstrates no significant quantitative changes in the aerobic and microaerophilic bacterial population belonging to this microbiota according to the reproductive status of these animals, although there were qualitative changes among the groups of animals studied.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Angiomyofibroblastoma of the vagina in bitch: case report

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Angiomyofibroblastoma is a rare benign mesenchymal neoplasm most commonly located in the vulva of women. The vaginal location is even rarer compared to the vulvar location, with few cases described in the human literature. The neoplasm was first described by Fletcher et al. (1), who observed differences between the benign type and angiomyxoma, an aggressive type of tumor. The most reported vaginal tumors in dogs are leiomyomas, fibroepithelial polyps, fibromas, among others, and are generally benign and do not cause metastasis. To date, there have been no reports of angiomyofibroblastoma in any species of veterinary medicine. A 14-year-old intact poodle bitch was conducted to the veterinary hospital presenting a prolapsed structure in the vagina. In the anamnesis, the owner informed that the structure had prolapsed a few hours ago, and exogenous hormones had never been used. During the physical examination, the bitch presented vital parameters considered normal for the species. On palpation and vaginoscopy, a pedunculated shape structure from vaginal mucosa was observed protruding from the upper vulvar region. The treatment was vaginal lumpectomy surgery, requiring an episiotomy to fully visualize the distal part of the peduncle, and ovariohysterectomy. The procedure was performed the day after the appointment since there were lesions on the vaginal mucosa exposed to the external environment. The specimen was sent for histopathological analysis, concluding the angiomyofibroblastoma diagnosis. Microscopic evaluation revealed the proliferation of mesenchymal cells organized in bundles and interspersed with intense collagenous stroma. These cells exhibited fusiform, poorly delimited, discrete, and eosinophilic cytoplasm. Nucleus oval to elongated, with dense chromatin and inconspicuous nucleolus. Discreet anisocytosis, anisokaryosis, and pleomorphism were noted, in addition to zero mitotic figures in ten higher magnification fields (2.5 mm^2). In the special Masson's Trichrome staining, the neoplastic cells stained red and blue (biphasic staining), which is incompatible with leiomyoma and fibroma. Furthermore, immunohistochemistry was performed for alpha-smooth muscle actin, with no neoplastic cell immunostaining. The vaginal lumpectomy treatment was effective and promoted complete resolution of the condition and the restoration of animal welfare.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Resveratrol attenuates doxorubicin-induced toxicity during *in vitro* culture of mouse isolated preantral follicles

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Doxorubicin, an antineoplastic agent used to treat a variety of tumors, can cause DNA damage in ovarian follicles and gonadotoxicity. The use of natural compounds with antioxidant properties, such as resveratrol, may attenuate doxorubicin-induced follicular damage [1,2]. The aim of this study was to evaluate whether resveratrol can inhibit or reduce doxorubicin-induced toxicity during *in vitro* culture of mouse isolated preantral follicles. In experiment 1, secondary follicles were isolated and cultured for 12 days in control medium (α-MEM+) or in α-MEM+ supplemented with doxorubicin (0.1 µg/ml) alone or associated with different concentrations of resveratrol (0.5, 2 or 5 µM). For experiment 2, follicles were cultured in α-MEM+ alone or supplemented with doxorubicin (0.3 µg/ml) or 5 or 10 µM resveratrol associated or not with doxorubicin (0.3 µg/ml). The endpoints analyzed were morphology (survival), antrum formation, follicular diameter, mitochondrial activity, glutathione (GSH) levels, and DNA fragmentation. In the first experiment, 0.1 µg/ml doxorubicin maintained survival and antrum formation similar to the control. Contrary to our results, Morgan et al. (2013) [3] reported increased percentages of degenerated follicles after culture of mouse ovarian tissue in medium supplemented with 0.1 µg/ml doxorubicin for up to 24 h. Therefore, it is suggested that the reduction in follicular viability caused by doxorubicin depends on the concentration and *in vitro* culture system used. Resveratrol at 5 µM alone increased survival, antrum formation and GSH levels, and maintained mitochondrial activity compared to the control. This may have occurred because resveratrol has antioxidant properties that help mitigate the harmful effects of oxidative stress under *in vitro* conditions, by increasing levels of the endogenous antioxidant GSH, and maintains the mitochondrial function of follicular cells [4,5]. In the second experiment, 0.3 µg/ml doxorubicin reduced survival, antrum formation, and follicular diameter compared to the control. Due to its non-specific action on cells, doxorubicin can harm non-cancerous cells, including ovarian cells, causing follicle atresia in different stages of development through increased oxidative stress [6]. Resveratrol at 10 µM associated with doxorubicin attenuated the damage caused by the antineoplastic by improving follicular survival and reducing DNA fragmentation. These results are in line with a previous *in vitro* study that showed that resveratrol (50 µM) increases the viability and reduces apoptosis of granulosa cells compared to cells cultured only with cyclophosphamide [5]. In conclusion, supplementation of the *in vitro* culture medium with 0.3 µg/ml doxorubicin reduced the survival and impaired the development of mouse isolated preantral follicles. Resveratrol at 10 µM reduced doxorubicin-induced follicular atresia by preserved DNA integrity.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

EFFECT OF *IN VITRO* SUPPLEMENTATION OF MATURATION MEDIUM WITH N-ACETYLCYSTEINE ON THE EXPANSION OF CUMULUS CELLS AND THE DEVELOPMENTAL COMPETENCE OF SHEEP OOCYTES

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Within the realm of *in vitro* production (IVP) of embryos, the *in vitro* maturation stage (IVM) stands out due to the heightened production of reactive oxygen species (ROS), which can compromise cellular integrity (1), necessitating the incorporation of antioxidants during IVM. N-acetylcysteine (NAC) serves as an antioxidant cysteine precursor that mitigates oxidative damage by restoring reduced glutathione (GSH) levels (2). Consequently, the aim of this study was to assess the impact of NAC on the *in vitro* maturation of sheep oocytes. To achieve this, sheep ovaries were retrieved from a local slaughterhouse, and cumulus-oocyte complexes (COC) were collected using a vacuum pump. The COC were categorized and evenly distributed into four groups: the CON group, lacking additional antioxidants, and the NAC1, NAC1.5, and NAC2 groups, each supplemented with 1 mM, 1.5 mM, and 2.0 mM NAC, respectively, within the same medium as the CON group. IVM was conducted for 24 hours at 38.5°C with 5% CO2. Following IVM, the matured oocytes were assessed for cell expansion and utilized for evaluating chromatin configuration. A separate subset of mature oocytes underwent *in vitro* fertilization (IVF) and were incubated for 20 hours at 38.5°C with 5% CO2. The presumed zygotes were then subjected to *in vitro* culture (IVC) at 38.5°C with 5% CO2 for 48 hours, followed by an assessment of the cleavage rate. The results underwent analysis using the Chi-square test, Kruskal-Wallis test, and Student-Newman-Keuls tests, with significance set at $P< 0.05$. In evaluating cumulus cell (CC) expansion, NAC1 exhibited a lower percentage of partial expansion and a higher percentage of total expansion compared to the NAC1.5 and NAC2 groups, without significant difference from the CON group, demonstrating that increased NAC levels induce a reduction in CC expansion due to a pro-oxidant effect (3). Both the NAC1 and NAC1.5 groups displayed a noteworthy increase in the number of oocytes in the metaphase II stage compared to the CON and NAC2 groups, attributed to the inhibitory effect of high NAC doses on the NF-κB (nuclear factor kappa B) pathway (4). The NAC1 group exhibited a higher number of cleaved structures and a greater percentage of embryos with more than 8 cells compared to the other NAC-supplemented groups, facilitating favorable embryonic development. In conclusion, a concentration of 1 mM NAC promotes greater expansion of cumulus cells, enhances metaphase II rates, and augments the cleavage rate.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

HIGH-PROTEIN DIET INTAKE ALTERS ESTROUS CYCLE DURATION, FOLLICULAR ATRESIA AND OVARIAN GENE EXPRESSION RELATED TO FOLLICLE DEVELOPMENT AND ATRESIA IN NULLIPAROUS SWISS MICE

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The consumption of high-protein diets is common among people with different exercise routines, but the diet is rarely monitored by a specialised professional and its effects on female reproductive health are currently unknown. Likewise, in livestock production this type of diet is mainly used to improve performance, although the effects on reproductive function are not well understood. Our aim was to evaluate the effect of high-protein diet intake on estrous cycle duration, folliculogenesis and ovarian gene expression in nulliparous females. Adult female Swiss mice (n=20) were divided into two experimental groups: NP- control diet, (14% crude protein, n=10); HP- high-protein diet, (55% crude protein, n=10). Both the diets were fed ad libitum for three weeks and simultaneously the estrous cycle was assessed daily by vaginal cytology. All females were euthanised on day 21 of treatment and distributed into four experimental subgroups according to the estrous cycle phase established prior to euthanasia: NPF- control diet and follicular phase; NPL- control diet and luteal phase; HPF- high-protein diet and follicular phase; HPL- high-protein diet and luteal phase. The ovaries were collected during necropsy for histomorphometrical evaluation of follicular density and evaluation of the ovarian expression of genes related to follicle development and atresia in each subgroup (CEUAº343/2022). The results were analyzed using the Tukey-Kramer mean comparison test with a significance value of $P< 0.05$. When assessing the estrous cycle, the HP group had a longer cycle length ($P= 0.031$), which was associated to a longer follicular phase ($P= 0.0049$) and a shorter luteal phase ($P= 0.0025$) when compared to the NP group. In the follicular phase, the HPF group showed a lower density and proportion of atresic follicles in the total primordial follicles (quiescent and active) (density: $P=0.05$; proportion: $P=0.02$), activated primordial follicles (density: $P=0.04$; proportion $P=0.04$) and secondary follicles (density $P=0.04$, proportion: $P=0.02$) when compared to the NPF group. In addition, secondary follicles density in the HPF group was lower than the NPF group ($P=0.04$). When the luteal phase was assessed, the HPL group showed lower density and proportion of secondary atresic follicles (density $P= 0.02$, proportion: $P= 0.047$); the remaining follicle density categories in this phase were not affected by previous nutritional treatment ($P> 0.05$). Regarding the expression of ovarian genes associated with follicular development and apoptosis, in the HPF group, gene expression of LHCGR, IGFIR, BAX, ACVR1, FSHR and CASP3 was up-regulated ($P< 0.05$). On the other hand, the expression of LHCGR, BAX, ACVR1 and FSHR was down-regulated in the HPL group ($P<0.05$). Our results suggest that prolonged consume of a high-protein diet alters the reproductive cycle, increasing the duration of the follicular phase and reducing the luteal phase, as well as reducing follicle atresia, especially in the follicular phase. Finally, consumption of a high-protein diet modulates, in a phase-dependent manner, the expression of genes associated with follicular development, differentiation and maturation, as well as genes associated with follicle atresia and apoptosis. These findings indicate that the high-protein diet has significant effects on modulating follicular development and ovarian function, although further studies on the effects of this diet on fertility and reproductive health are essential.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

The risk of antipsychotic aripiprazole exposure in female reproduction

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Aripiprazole (ARPZ) is a drug used to treat schizophrenia and mental disorders by stabilizing the dopaminergic system. Studies on psychotropic drugs have demonstrated negative effects on hormonal regulation and sexual behavior, a consequence of their mechanism of action, but their potential gonadotoxic effects have not yet been fully clarified. In this way, we evaluated the possible adverse effects of ARPZ exposure on the reproductive system of female Wistar adults rats. This study was approved by the Ethics Committee of UERN 006/21 and the Ethics Committee of UFERSA 23091.014948/2019-20. Adult female Wistar rats (n=12/group) from control group (CTRL), group treated with 0.3mg/kg (EXP1), 3.0mg/kg (EXP2) 6.0mg/kg (EXP3) of aripiprazole were treated for 21 days and the estrous cycle of the rats was monitored. The control group received vehicle solution in the 1ml/kg of volume (Dimethylsulfoxide – DMSO + saline solution) and the treated groups received aripiprazole diluted in vehicle solution in the same volume. The treatment was performed by gavage. At the end of the exposure, six females in estrus from each experimental group were subjected to sexual behavior assessment (lordosis quotient). After the test, the females were kept with the respective male until the following morning, and the vaginal wash was performed to confirm the presence of sperm (gestational day - GD01). On GD20, females were euthanized by anesthetic saturation and blood samples were collected for hematological evaluation. Fertility and fetal development were assessed through laparotomy and data collection: weight of the pregnant uterus and fetuses, sexing, number of corpora lutea, implantation and reabsorptions points. Pre- and post-implantation loss rates, placental index and fertility potential were determined. The other 6 females per group were euthanized at the end of treatment and their ovaries and uterus were fixed for histopathological analysis. Data were evaluated using ANOVA, followed by Dunnett or Kruskal-Wallis test, followed by Dunn ($p<0.05$). A significant reduction in serum albumin levels and an increase in triglyceride levels were observed in EXP3 treatment when compared to CTRL. No significant differences were found regarding histomophometric parameters. However, the number of estrus in the EXP3 group was significantly lower, as was the lordosis quotient, when compared to the CTRL group. No changes were observed in the fertile potential and pre- and post-implantation loss rates, but when comparing the weights of the placentas, there was a significant increase in the EXP03 group compared to the CTRL. Several compounds can alter female reproductive function, acting directly on the system or indirectly, via changes in the endocrine system. Thus, we can observe that, based on this experimental model, that aripiprazole has impacts on the female reproductive system. New studies must be carried out in order to evaluate the impact of placental enlargement on fetal development. The authors would like to thank the grants to PIVIC UFERSA Scholarship and UFERSA/PROPPG PP (grant number 23091.014593/2019-02) and PIAP (grant number 23091.005800/2023-42).

Animal Reproduction

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FEMALE REPRODUCTIVE BIOLOGY

Use of ultrasound for gonadal maturation assessment and sex determination in *Prochilodus brevis*

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The Brazilian bocachico fish, (Prochilodus brevis), is endemic on region of northeast Brazil and highly consumed by the local population. It is a rheophilic fish, that is, during its reproductive process they carry out a migration that is essential for the completion of oocyte maturation and ovulation. Therefore, in captivity, they require exogenous hormonal therapies to enable reproduction (1). One of the challenges encountered in breeding this species in captivity is the absence of apparent sexual dimorphism, necessitating the use of existing sexing methods, which are considered invasive. Thus, ultrasonography emerges as a non-invasive, rapid, and efficient alternative for sexual determination in this species (2). Proper sexing and monitoring of gonadal development, especially in females, enable better selection of breeders, thereby contributing to aquaculture development (3). Therefore, the main objective of the study is to establish an ultrasonographic sexing method for Brazilian bocachico fish. The experiment occurred in two stages, one within the reproductive season in February 2018, and one outside the season in August of the same year. considering the breeding season of the species. A total of 18 Brazilian bocachico fish from a breeding stock in Canindé-CE were selected,. identified using microchips, and kept for two months in a tank with a permanent recirculation system at the State University of Ceará, in Fortaleza. During this period, they were fed with commercial maintenance feed. The temperature, pH, ammonia and nitrite were systematically monitored and water was exchanged so as not to compromise the welfare of the animals. Within the reproductive season, eleven females were analyzed ultrasonographically. Fish were manually restrained in dorsal recumbency, fully submerged in water and without sedation. The animals were evaluated from various angles, generating longitudinal and transverse images. Outside the season, seven females were analyzed. However, due to the difficulty of obtaining images, ultrasonographic analysis was performed out of the water. For this, the animals were sedated in a solution containing 318 mg of Eugenol ReagentPlus® per liter of water until they lost balance. During this analysis, appropriate carboxymethylcellulose-based gel was used, and the examination sequence was analogous to the first occasion. Images from both cuts were digitally captured and stored in JPEG format. In the first stage, ultrasonographic analyses were performed using portable equipment (Logic E, General Electric, http://www.ge.com). In the second stage, fixed equipment (Mylab 40, Esaote, http://www.esaote.com) coupled with 12MHz linear transducers was used, and gain was adjusted to obtain the best possible images. On both occasions, two-dimensional B-mode ultrasonography was performed in resolution mode. Images were saved on a flash drive, transferred to a microcomputer, and evaluated using ImageJ® software. It was observed that ovaries had larger measurements within the reproductive season than outside it. Within the reproductive season, it was not possible to individualize the gonads due to the proximity between the right and left gonads. Additionally, there was a significant difference in the images obtained within the reproductive season, where it was possible to visualize type V (larger) and type II (smaller) oocytes, confirming that the females were in the mature stage. Outside the reproductive season, type II, III, and IV oocytes were visible, indicating that the animals were in the maturation stage. These results align with the histological study of the gonads of this species over time conducted by (4). Thus, it is concluded that ultrasonography can be used as a non-invasive tool for Brazilian bocachico fish sexing, causing minimal stress and without affecting their reproduction.

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FEMALE REPRODUCTIVE BIOLOGY

BIRTH RATE AND MORTALITY RATE OF LAMBS ON FARMS IN THE GUARAPUAVA-PR REGION

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Brazil has a sheep herd of approximately 20 million heads, which is expected to increase due to the growing demand for meat from these animals in Brazilian and global cuisine. This highlights the importance of maintaining effective reproductive health and avoiding herd losses (1, 2). Lambing rates, fertility, prolificacy, and reproductive efficiency are important indicators of the reproductive performance of the sheep herd. The birth rate, influenced by management and reproductive factors, also requires attention, as does the weaning rate, which reflects the survival of lambs (3, 4). In this context, this research focused on monitoring sheep farming in the region of Guarapuava - PR, in order to evaluate the birth and mortality rates of lambs, with the aim of improving the reproductive performance and productive efficiency of these farms. The present study was developed on farms in the city of Guarapuava – PR and region, intended for raising sheep, which were monitored by a veterinarian responsible for the reproductive management of the animals. Initially, visits were carried out and five farms were selected that had zootechnical records so that calculations of the herd's reproductive indices could be carried out. The animal breeds were Texel, Ile de France, Ideal and crosses. On all farms, the extensive breeding system was used (winter oat or ryegrass pasture and Tifton summer pasture), with mineral supplementation in the trough and water ad libitum. The results obtained for the birth rate were 123%, 118.64%, 126.37%, 115.38% and 135.29% for farms A, B, C, D and E, respectively. The acceptable birth rate for sheep varies between 80 and 90% (5), in the farms monitored, values were above the average described in the literature. Rates that exceeded 100% indicate that the ewes in the flock had double births. The birth rate of the five farms exceeded 100%, since there were 15 double births on farm A, 16 on B, 12 on C, 2 on D, and 3 on farm E. The weaning rate was 88.23%, 94.28%, 92%, 100% and 86.95% for farms A, B, C, D and E, respectively. According to the literature, the mortality rate for lambs until weaning varies between 5% and 8% (6). In properties A, B, C and E mortality rates were 13.3%, 6.06%, 4.34% and 15%, respectively, while farm D kept all lambs born until weaning, with a rate 0% mortality rate. Farms A and E presented results above the average, farm B within the average and farm C below the expected average (6). The results obtained show a birth rate within the average described in the literature, indicating the occurrence of double births on farms and, consequently, an increase in the number of lambs born per season. Furthermore, the mortality rate was above average on two farms, indicating the need to review the management practices adopted during this phase of the animals' lives, with the aim of reducing losses and increasing the financial return on production.

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FEMALE REPRODUCTIVE BIOLOGY

Influence of different concentrations of eCG in the *in vitro* maturation medium of ovine oocytes

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In vitro oocyte maturation (IVM) stage induces changes that enhance oocyte competence (1). To ensure these changes and the expression of receptor genes present in the granulosa cells, gonadotrophins (2) are added to the maturation medium. In addition to mimicking FSH, eCG stimulates LH receptors and activates intracellular messengers that regulate cellular response (3). However, few studies have examined different concentrations of eCG in the IVM medium in sheep. This study aimed to evaluate the effect of varying concentrations of eCG in the IVM medium on the IVP of ovine embryos. Ovine ovaries from a local slaughterhouse were used, and oocytes were harvested using a vacuum pump at a pressure of 20 mmHg and an 18G catheter. After collection, the oocytes were randomly divided into groups of 10-15 and subjected to IVM in 75 µL drops of medium according to the following treatment groups: eCG5, composed of TCM-199 supplemented with 500 IU penicillin, 0.5 mg streptomycin, 1.25 µg amphotericin B, 0.2 mM sodium pyruvate, 10% (v/v) fetal bovine serum (FBS), and 5 IU eCG; and the eCG10, eCG15, and eCG20 groups, using the same medium as the eCG5 group but with 10, 15, and 20 IU of eCG, respectively, replacing the 5 IU of eCG. The drops were coated with mineral oil and incubated for 24 hours at 38.5°C with 5% CO2. Subsequently, the oocytes were stained with Hoechst 33342 and assessed for chromatin configuration using an epifluorescence microscope. The results were expressed as percentages and subjected to the Chi-square test in the Epi Info software (Epi Info 7.2.5, Atlanta, GA, USA, 2021), with a significance level of 5%. Analysis of the oocytes' chromatin stage revealed no significant difference between the treatment groups for the germinal vesicle (GV) and germinal vesicle breakdown (GVB) stages. However, for the metaphase I (MI) stage, the eCG15 group was inferior to the others, while for the metaphase II (MII) stage, the same group surpassed the eCG5 and eCG10 groups. Thus, the higher rate of oocytes in MII observed in the medium supplemented with 15 IU of eCG (37.7%) may result from the interaction between FSH and LH, present in this medium (4), at a higher concentration compared to the groups containing 5 and 10 IU. Lower eCG concentrations (groups eCG5 and eCG10) are likely to result in less interaction of FSH and LH present in the IVM medium, leading to lower rates of MII. Therefore, this study concludes that increasing the eCG concentration, as in the group containing 15 IU, can promote the *in vitro* maturation of ovine oocytes by increasing the rate of oocytes in MII.

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FEMALE REPRODUCTIVE BIOLOGY

ISOLATION AND CHARACTERIZATION OF GOAT OVIDUCT FLUID-DERIVED EXTRACELLULAR VESICLES THROUGH THE ESTROUS CYCLE: A PHYSICO CHEMISTRY REPORT

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Extracellular vesicles (EV) are nanoparticles formed by phospholipid membrane which are present in several biological fluids. These vesicles are responsible for the molecular exchange through intercellular communication process [1]. The EV are often classified as microvesicles (100–1000 nm) or exosomes (30 –150 nm), which are currently reported by several studies as important components of murine, feline, bovine, and swine oviduct fluid (OF) [2]. However, there is no evidence about characterization of caprine oviduct EV (coEV). Thus, the aim of this study was to isolate and characterize, coEV. Goat oviducts (n=21) were obtained at local slaughterhouse and used to OF recovery, of which 10 were collected during follicular phase and 11 during luteal phase of estrous cycle, and dry transported to the laboratory at 4 ºC. The follicular phase was characterized by the presence of at least four antral ovarian follicles (3-8 mm) in the cortical region of the ovary. The luteal phase was characterized by the presence of a rosy corpus luteum with a diameter ≥ 6 mm. Each oviduct was flushed with 150 µL of phosphate-buffered saline (PBS) to maximize the recovery of OF. Then, the OF samples were centrifuged twice: at 300 g and 12.000 g for 15 minutes at 4 ºC and EV were later isolated from caprine OF by the serial ultracentrifugation method. Each OF individual sample from follicular and luteal phase were ultracentrifuged twice at 100,000 g for 90 minutes at 4 ºC. The final pellet was resuspended in 150 µL of PBS, aliquoted and stored at –80 ºC until EV characterization. The EV characterization was performed by Dynamic light scattering (DLS) using Zetasizer Nano ZS to measure size distribution, polydispersity index (PDI), and zeta potential (ZP). The transmission electron microscopy (TEM) was conducted to confirm the presence and characterize the morphology of EV in caprine OF. In addition, dot blotting (DB) were used to detect the presence of exosomal markers proteins (CD63 and HSPA1A) in both estrous cycle phases. The size of the oEV collected during the follicular phase (274.8 + 12.1 nm) was significantly lower ($P<0.05$) than oEV during the luteal phase (345.4 + 14.5 nm). Similar results were observed for the PDI, where the follicular group polydispersity (0.29 + 0.01) was significantly lower than luteal group (0.39 + 0.02). However, no statistical differences were observed for the ZP between follicular (-13.2 + 0.26) and luteal phase (-12.4 + 0.49). The TEM analysis identified the presence of vesicles with diameter ranging 150-350 nm, similar to microvesicles, and exosome like vesicles with diameter ranging 50-150 nm presenting a cup shape morphology. Additionally, the DB assay showed the presence of CD63 and HSPA1A in oEV and OF samples. These data lead us to believe that the influence of the estrous cycle on the production of oEV is different among species such as murine, swine, bovine, and caprine, which may be attributed to their physiological peculiarities [3]. In conclusion, our report about the presence and characterization of coEV, is the first step to study the involvement of these microvesicles and exosomes on the goat reproductive events and opens the possibility of their use as a tool to improve different *in vitro* reproductive biotechnologies.

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FEMALE REPRODUCTIVE BIOLOGY

FIBER BLEND SUPPLEMENTATION IMPROVES FOLLICLE NUMBER AND STEROIDOGENIC CAPACITY IN GILTS

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Introduction: Achieving litter uniformity is a significant challenge in pig farming, yet the underlying biological mechanisms remain incompletely understood. Studies indicate that factors such as pre-ovulatory follicle size and oocyte maturation strongly influence this outcome. Fiber supplementation emerges as a promising approach, as studies have demonstrated that supplementary fiber can enhance oocyte quality and litter uniformity. Hence, this study aimed to assess the impact of fiber supplementation throughout the second estrous cycle on follicle morphology and estradiol secretion. Materials and Methods: The experiment was conducted at the Swine Research Laboratory of VNP - FMVZ/USP, using 40 pubertal commercial hybrid gilts. These gilts, matched for age, body weight and backfat thickness, were randomly allocated into two experimental groups: CON, which received a standard growth diet (n = 20), and FIB, which received the same diet supplemented with fibrous additives (n = 20). Both diets were adjusted to provide similar metabolized energy. The fibrous supplement consisted of a blend of wheat meal, lignocellulose, citrus pulp, and guar gum. Supplementation lasted for 19 days, starting on the onset of the second estrous cycle. Ultrasound monitoring of follicular development was conducted throughout the cycle. On the 20th day of supplementation, animals were euthanized, and the ovaries collected and processed for morphological evaluation (n=10 females from each experimental group). Morphological assessment involved categorizing and quantifying follicles based on diameter (follicles < 2mm, 2-4mm, 4-6mm and > 6mm), and histological parameters (quiescent primordial; activated primordial; primary; pre-antral; secondary, and tertiary) to further determine follicle density (number of follicles/μm2). Moreover, atretic follicles were determined by the presence of pycnotic nuclei, nuclear regression or detachment of the granulosa cells from the basal membrane and then quantified. In addition, blood samples were collected from the jugular vein to determine serum 17β-estradiol levels in duplicate using a commercial ELISA kit (Cayman Chemical, Inc., Ann Arbor, MI, USA), according to the protocol provided by the manufacturer. Results: Even though fiber supplementation did not affect ovarian weight($P> 0.05$), higher number of follicles on the ovarian surface were observed ($P<0.05$). Among those, greater proportion of follicles greater than 6 mm diameter were noticed in FIB compared to CON females($P<0.05$). Although follicle densities and oocyte areas were similar between experimental groups, FIB gilts exhibited larger granulosa layer areas and higher plasma levels of 17β-estradiol($P<0.05$). Discussion: The fiber blend used in the present study seems to positively affect preovulatory follicles' size, likely through its estrogenic action, as evidenced by increased granulosa cell area and elevated 17β-estradiol levels. This improvement in follicular size suggests potential benefits for reproductive outcomes, although further studies to explore its effects on parameters such as litter size and uniformity are necessary.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

CHARACTERIZATION OF THE REPRODUCTIVE SEASON FOR BREEDING SHEEP ON FARMS IN THE GUARAPUAVA – PR REGION

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Reproduction is characterized as an important ally to increase sheep production, however, for this activity to be effective, management practices that aim to improve the animals' reproductive performance must be adopted, as it is characterized as a serious problem in Brazilian sheep farming at low levels. reproductive efficiency. These practices, when well applied, generate greater profitability, enabling greater selection pressure, thus replacing the less productive. Reproductive monitoring of herds is one of the most important tools for successful production. The main reproductive indices that must be analyzed are pregnancy rate, birth rate, twin birth rate, in addition to birth and mortality rates (1, 2). In view of the above, the present study aimed to monitor sheep farming in the city of Guarapuava - PR and region to characterize the reproductive season of these herds. The present study was developed on farms in the city of Guarapuava – PR and region, intended for raising sheep, which were monitored by a veterinarian responsible for the reproductive management of the animals. Initially, visits were carried out and five farms that had zootechnical records were selected to characterize the reproductive season could be carried out. In all five farms that presented notes on the reproductive management of animals, the extensive breeding system was used (winter oat or ryegrass pasture and Tifton summer pasture), with mineral supplementation in the trough and water ad libitum. The animals were of the Texel, Ile de France, Ideal and cross breeds. The duration of the breeding season varied across the five properties monitored, being 50 days on farms A and B, indicating greater selection pressure on sheep and breeders, while on property C it was 60 days, on D it was 90 days and on E 210 days. In the literature, the duration of the breeding season for sheep should be approximately 51 days, that is, which corresponds to the use of three estrous cycles (3), therefore, farms C, D and E lasted longer than recommended. All farms had natural breeding as a reproductive method, with farm A having 104 sheep for two rams (proportion 1 male/52 dams), farm B 79 females for one ram (1/79), farm C with 98 females and two males (1/49), D with 36 females and two rams (1/12), and E with 22 females and one ram (1/22). The male/female ratio, the literature recommends for breeding with extensive management, using one ram for 25 to 30 sheep (3). Farms A, B and C had a male/female ratio greater than recommended, while farms D and E had a lower than recommended ratio. Reproductive biotechniques, such as artificial insemination or fixed-time artificial insemination, were not used on the monitored properties. The pregnancy rate was 62.5% (65/104) on farm A, 74.68% (59/79) on farm B, 100% (98/98) on farm C, 36.11% (13/36) on farm D) and 77.27% (17/22) on farm E. According to the literature, the average pregnancy rate of sheep with extensive management, with a breeding season of 60 days is of 81.16% (4). Farms B and E presented values close to those described by the authors, farm C a higher value, while A and B below expectations. Many factors can affect the pregnancy rate, such as adequate selection of matrices and breeders, length of the breeding season, male/female ratio, body score of the sheep, among others. Collecting these data revealed a complex and diverse picture of reproductive season in these herds. Understanding these variations may help in the implementation of more effective reproductive management strategies in sheep herds, which is why more studies are needed in order to improve reproductive rates and, consequently, increase production.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Influence of co-culture with bovine oviduct spheroids on the lipid profile of bovine embryos

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Bovine IVP is a well-established tool, but that does not yet completely mimic the *in vivo* environment. Indeed, embryos developed *in vitro* contain excess lipids, which is known to be detrimental to their survival after cryopreservation. In the *in vitro* conditions, factors such as oxygen tension are known to affect embryo quality. It was recently demonstrated that the co-culture of bovine embryos with bovine oviduct epithelial spheroids (bOES) improved embryo development. However, it is still unknown if the bOES affect the lipid content of co-cultured blastocysts. This study investigated the lipid profile of IVP bovine embryos cocultured with bOES at different oxygen tensions. Periovulatory stage oviducts were obtained from Bos taurus cycled cows at a local slaughterhouse, and oviduct cells were cultured to produce bOES. On the third day of cell culture, bOES measuring 100-200 µm were selected for co-culture with zygotes. In parallel, the ovaries were aspirated, and Grade 1 and 2 COC were selected and subjected to *in vitro* maturation (50-80 COC/well) for 22-24 h under 5% CO2 in air at 38.8 °C. Frozen-thawed spermatozoa (1x106 spz/mL) from Holstein bulls were selected by Percoll and used for IVF (50-80 COCs/well), for approximately 22 h under 5% CO2 in air at 38.8 °C. For the *in vitro* development (IVD), four groups were formed; varying the oxygen tension (5% O2 vs. 20% O2), and the presence or not of bOES [control at 5% (C5), bOES at 5% (B5), control at 20% (C20), or bOES at 20% (B20)]. In both bOES groups, there was an equal amount (1:1, maximum of 25:25) of presumptive zygotes and bOES in the IVD droplets (25 µL) of modified SOF medium, at 38.8 °C. The cleavage rate was evaluated on the second day (D2), the blastocyst rate on D7-8, and the hatching rate on D8 (nb of hatching embryos/nb of blastocysts). Blastocysts (D8) from the four groups (10-13/group) were evaluated individually and directly by MALDI-TOF mass spectrometry using a RapifleX MALDI Tissue typer TOF mass spectrometer (Bruker Daltonics, Germany) equipped with a Smartbeam 3D Nd: YAG laser (355 mm). Oil red staining was used to quantify embryo lipid droplets (n=6/group), and images were analyzed by Image J. Data treatments and processes were performed using the MALDIquant and MALDIquantForeign (v 1.17 & v0.11.1) packages of the R software (version 3.5.0) (Gibb and Strimmer, 2012). Non parametric statistical analyses were applied using Kruskal-Wallis and Wilcoxon tests. The results showed high coefficients of variation (>30%), but after sorting the samples, they all showed a CV <50%. The analysis of the principal components of all masses showed a similar profile between the groups. However, two unidentified masses (605.58 m/z and 603.56 m/z) were significantly different ($p<0.01$) between the C20 and B20 groups. Of note, oil red staining of blastocysts showed a reduction in lipids in the C5 compared to the C20 group. In conclusion, the reduced amount of lipids in the C5 group and the detection of differential masses by MALDI-TOF between the C20 and B20 groups indicate, respectively, an effect of oxygen tension and bOES on lipid metabolism. The non-reduction of lipids in the bOES groups may indicate a role of bOES in supplying lipids to the embryo. Recently, the effect of bOES on genes involved in lipid metabolism has been confirmed by the RNA-seq analysis of blastocysts (in prep.). Finally, the high CV found in the mass spectrometry analysis suggests this technique might be adapted to become more reliable for analyzing whole embryos. Financial support: CAPES (code 001), CNPq, and FAPERJ.

Keywords: bOES, lipid content, spectrometry, embryo, bovine.

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FEMALE REPRODUCTIVE BIOLOGY

Oviduct epithelial spheroids during *in vitro* culture enhance bovine embryo development, mitigate the negative impact of oxidative stress and change the embryonic transcriptome

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The mammalian oviduct provides an optimal environment for early embryo development. On the contrary, *in vitro* embryo production exposes the embryos to oxidative stress with deleterious effects on blastocyst development and quality. Considering the antioxidant capacity of the oviduct epithelium, we developed an *in vitro* model co-culturing bovine oviduct epithelial spheroids (OES) and embryos. We hypothesized that co-culture of OES with *in vitro* produced embryos up to the time of embryonic genome activation (5 days) or blastocyst stage (7-8 days) could mitigate the effects of oxidative stress conditions. Oocytes were collected from bovine ovaries from a slaughterhouse and *in vitro* matured for 22 h. Then, oocytes were fertilized (day 0) using frozen-thawed Percoll-washed bull semen at 38.8°C. After 24h (day 1), groups of 25 presumptive zygotes were *in vitro* cultured in 25 µL droplets of SOF medium with 5% fetal calf serum, at 38.8°C, 5% CO2, and under 5% or 20% O2, with or without OES up to day 5 or day 8 of culture, as follows: 1) controls-5%; 2) controls-20%; 3) 5dOES-5%; 4) 8dOES-5%; 5) 5dOES-20%; and 6) 8dOES-20%). Cleavage rates were evaluated on day 2 and blastocyst rates on days 7-8. Expanded blastocysts on days 7-8 were either evaluated for total cell numbers (Hoechst staining) and for gene expression analysis by Illumina RNA-sequencing. Embryo development rates were compared by ANOVA, Tukey's post-tests. RNA-seq data was processed to remove adapter sequences and low-quality bases. Differentially expressed genes (DEGs) were identified with EdgeR (FDR≤1%). Functional analysis of DEGs was performed using Metascape. No differences were found on cleavage rates among the different culture conditions (73-81%, 8 replicates). Under 5% O2, the presence of OES up to day 5 or day 8 did not affect blastocyst rates compared to controls without OES (27-32% vs. 30% on day 8) but increased the number of cells per blastocyst when OES were co-cultured for 8 days (137.6 ± 10.8 vs. 102.6 ± 8.4 cells; $P<0.05$). Under 20% O2, blastocyst rates were significantly higher in the presence of OES compared to controls without OES (30.7% and 31.8% in 5dOES-20% and 8dOES-20% vs. 19.8% in controls-20%; $P<0.05$). Furthermore, cell numbers per blastocyst were significantly increased compared to controls with no difference between 5 and 8 days of co-culture (112.7 ± 7.8. and 138.1 ± 10.5 cells, respectively, vs. 82.1 ± 4.5 cells in controls; $P<0.0001$). Under 5% O2, 568 and 911 DEGs were identified in blastocysts co-culture with OES up to day 5 and 8, respectively, compared to controls. Under 20% O2, 559 and 1282 DEGs were identified in blastocysts co-cultured with OES up to day 5 and 8, respectively, compared to controls. The most enriched pathways in which DEGs were involved included lipid metabolism, cell-cell junction, intracellular transport, membrane organization, and oxidoreductase activity. In conclusion, OES co-culture improved embryo quality in all groups and mitigated the negative impact of high oxygen conditions on *in vitro* embryo development. OES co-culture up to day 5 of development, i.e. up to the embryonic genome activation, was enough to induce this beneficial effect. Finally, the OES altered the embryonic transcriptome with highest impact under oxidative stress conditions, evidencing for the first time a modulation of the embryo-OES dialog according to the embryonic environment.

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FEMALE REPRODUCTIVE BIOLOGY

Grape residue extract enhances mitochondrial function and promotes meiotic resumption of sheep oocyte from *in vitro*–grown secondary follicles

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In vitro culture systems of preantral follicles are important for providing numerous oocytes to be used for *in vitro* maturation and fertilization (1). However, oxidative stress induced by *in vitro* conditions may lead to the production of poor-quality oocytes (2,3). In this context, there is a growing interest in the use of natural products to prevent oxidative damage and promote follicular development *in vitro*, such as grape residue extract. This study was conducted to evaluate the effects of adding of acidified extract of grape industrial residue (Vitis vinifera cv. Syrah) as a supplement to the base medium for *in vitro* culture of isolated sheep secondary follicles. Secondary follicles were isolated and cultured for 12 days in α-minimum essential medium (α-MEM) supplemented with bovine serum albumin, insulin, glutamine, hypoxanthine, transferrin, selenium, and ascorbic acid (control medium: α-MEM+) or in α-MEM+ supplemented with various concentrations of grape residue extract (0.1, 0.2, or 0.4 mg/ml). Follicular morphology, antral cavity formation, follicular and oocyte diameter, glutathione (GSH) levels, mitochondrial activity, DNA fragmentation and meiotic resumption were evaluated. After 12 days of culture, there was no difference ($P > 0.05$) among the treatments regarding morphology, antral cavity formation, follicular diameter, percentage of fully grown oocytes (≥ 110 µm), and DNA fragmentation. The GSH levels were similar ($P > 0.05$) among the α-MEM+, 0.2, and 0.4 mg/ml grape residue extract groups. Nevertheless, oocytes from secondary follicles cultured in 0.4 mg/ml grape residue extract showed higher ($P < 0.05$) mitochondrial activity and greater meiotic resumption than oocytes cultured in the control medium. The grape residue extract is rich in phenolic compounds with antioxidant properties, such as luteolin, which increased the rates of first polar body extrusion and porcine blastocyst formation by reducing intracellular ROS (4), and quercetin, which resulted in more MII in goat oocytes by increasing mitochondrial activity (5). Thus, it can be suggested that the compounds present in 0.4 mg/mL grape residue extract may act to improve mitochondrial activity, contributing to higher rates of meiotic resumption of oocytes from *in vitro*–grown secondary follicles. In conclusion, the supplementation of the base medium with 0.4 mg/ml grape residue extract maintained survival, improved mitochondrial activity, and promoted the meiotic resumption of oocytes from sheep secondary follicles cultured *in vitro*.

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FEMALE REPRODUCTIVE BIOLOGY

Administration of recombinant bovine somatotropin (rbST) at the beginning of a superovulatory treatment of Santa Ines ewes increases the cell number in *in vivo*-produced blastocysts

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Somatotropin is a metabolic hormone that stimulates the development and growth of ovarian follicles in ruminants. Therefore, the objective of the present study was to evaluate the effect of administering recombinant bovine somatotropin (rbST) at the beginning of a superovulatory treatment of Santa Inês ewes on *in vivo* embryo production, gene expression (AQP3, BAX, BCL2, CDH1, CDX2, HSP70, PRDX1, and SIRT2), mitochondrial activity, reactive oxygen species (ROS), and total number of blastomeres. For this, 40 Santa Inês ewes superovulated with 333 IU of pFSH (Pluset, Biogénesis Bagó, Curitiba, Brazil) were randomly distributed to receive 50 mg of rbST (ST group; n=20) subcutaneously together with the first of six decreasing doses of pFSH (25%, 25%, 15%, 15%, 10% and 10%) or did not receive rbST, remaining as controls (Control group; n=20). The animals underwent an ultrasound exam on the eighth day after mating to determine the total number of corpora lutea (CL) before the non-surgical embryo collection. The recovered blastocysts were dry-frozen for gene expression analysis by RT-qPCR or stained with Mitotracker Green (Invitrogen™, Waltham, MA, USA - M7514), 2',7'-dichloro-dihydro-fluorescein diacetate (H2DCHFDA, Invitrogen ™, D399) or Hoescht 33342 (Invitrogen™, D399) to determine mitochondrial activity, ROS levels, and total number of blastomeres. The results are presented as mean ± SEM, and differences were considered significant when P≤0.05 in the t-test (parametric data) or Mann-Whitney test (non-parametric). Administration of rbST did not affect the total number of CL (9.4 ± 1.1 vs. 9.3 ± 1.0), recovered structures (5.7 ± 1.4 vs. 4.5 ± 1.1), and viable embryos (3.8 ± 1.2 vs. 3.0 ± 1.0), as well as the mitochondrial activity (212 ± 102 vs. 255 ± 114 AU), and ROS (127 ± 46 vs. 195 ± 76 AU), in Control and ST ewes, respectively. However, administration of rbST enhanced the number of blastomeres in embryos at the blastocyst stage (79.80 ± 12.42 vs. 58.17 ± 6.49), and reduced the expression of the anti-apoptotic gene (BCL2) and the gene related to oxidative stress (PRDX1), with no other genes affected. Overall, the administration of rbST at the beginning of a superovulatory treatment did not affect embryo yield. On the other hand, although the rbST did not induce changes in the embryo's morphology, it positively affected other parameters of embryo quality, such as the number of blastomeres and expression of quality genes, which may benefit its probability of survival after transfer.

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FEMALE REPRODUCTIVE BIOLOGY

Anethole supplementation during *in vitro* culture of early antral follicles increases extrusion rates of oocytes and reduces area of the extruded follicles

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The oocyte extrusion is a phenomenon similar to ovulation that occurs spontaneously during the *in vitro* culture of ovarian follicles. This phenomenon has been previously reported during the *in vitro* culture of isolated preantral [1] and early antral follicles (EAF-[2]), being stimulated by the presence of factors added to the culture medium, such as hormones (insulin-[3], FSH-[4]). Natural compounds added to the base culture medium, such as anethole, have also significantly increased the occurrence of extruded follicles during the *in vitro* culture of EAF, especially at the end of the third third [2]. However, it is currently unknown whether the area of the resulting extruded follicles cultured in the presence of anethole differs from the control in its absence. Thus, the objective of the present study was to evaluate the effects of anethole addition during the *in vitro* culture of EAF on follicular morphological parameters and to verify if there is a correlation between the area of extruded follicles and the extrusion rate. To this end, ovaries were collected from goats (n=40) at local slaughterhouses and transported to the laboratory in minimum essential medium (MEM) supplemented with Hepes buffer (MEM-Hepes) at a temperature of approximately 4°C. In the laboratory, the ovaries were washed and then fragmented with the aid of a scalpel and forceps to obtain fragments of ovarian cortex with a thickness of approximately 1-2 mm thick. Manual isolation of EAF measuring between 300 and 350 µm was performed from the fragments under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) with the aid of 26-gauge needles attached to 1 mL syringes. The isolated follicles were selected for morphology, with all being classified as normal, presenting a translucent antral cavity and a clear oocyte without signs of degeneration, and cultured individually in 60×15 mm plates submerged in mineral oil for 18 days at a temperature of 38.5 °C and in an atmosphere with 7.5% CO2 containing 100 µL drops of α-MEM medium (ref. M5650, pH range of 7.2-7.4) supplemented with 3 mg/mL BSA, 5.5 µg/mL transferrin, 5 ng/mL selenium, 2 mM glutamine, 2 mM hypoxanthine, 0.911 mM/L pyruvate, 50 ng/mL GH, 10 ng/mL insulin and 100 µg/ml ascorbic acid, referred to as α-MEM+ (standard medium – control treatment), or α-MEM+ without ascorbic acid and supplemented with 300 µg/mL anethole (anethole treatment). Partial medium replacement was performed every two days in each drop. At the end of the culture, the follicles were classified as morphologically intact, extruded, or degenerated, the diameter of intact follicles was measured, and the area of extruded follicles was calculated. This experiment was replicated 4 times, and a total of 260 follicles were used. Despite not presenting differences in the rates of intact, degenerated follicles, and follicular diameter after *in vitro* culture ($p>0.05$), and not observing a correlation between the area of extruded follicles and the follicular extrusion rate ($r = 0.22$ $p>0.05$), the addition of anethole to the culture medium of EAF significantly increased the rate of extruded follicles ($p<0.05$). In addition, the extruded follicles from the anethole treatment showed a smaller area compared to the extruded follicles in the control treatment ($p<0.05$). Anethole supplementation has been previously associated with increased follicular extrusion rates during the *in vitro* culture of EAF [2]. In conclusion, in the present study, the smaller area in the extruded follicles in the anethole treatment suggests that this compound may induce early oocyte extrusion in follicles of smaller diameter, without affecting the viability or diameter of intact follicles.

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FEMALE REPRODUCTIVE BIOLOGY

Effect of the BNT162b2 vaccine against SARS-COV-2 on the fertility and pregnancy of female Wistar rats

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The Covid-19 pandemic, caused by the Sars-cov-2 virus, has been one of the biggest public health challenges faced by humanity in recent years and has triggered a global race to develop safe and effective vaccines. Among the vaccines developed, BNT162b2, a messenger RNA (mRNA) vaccine, has stood out for its effectiveness in preventing Sars-Cov-2 infection. The safety and effectiveness of vaccines are essential aspects to be evaluated, especially in vulnerable groups, such as pregnant women and their descendants. The administration of vaccines during pregnancy is a topic of interest and concern, raising questions about possible repercussions on fetal development and reproductive health. In this context, the present study aimed to evaluate the possible effects of the BNT162b2 vaccine on the pregnancy rate of Wistar rats and identify possible changes in the development of the offspring. The study was approved by the Ethics Committee under protocol number 002/2022-UERN. Wistar rats were used, 18 females and 9 males (for mating). The experimental design was divided into a Control group (n=9 females) treated with saline solution, administered intramuscularly. The Experimental group (n=9 females) received the BNT162b2 vaccine at a dosage of 30µg mRNA/dose intramuscularly. The doses were administered 21 days and 14 days before the start of mating and after pregnancy was confirmed on gestational days (DG) 12 and 18, totaling 4 doses. After administration of the vaccine, in the first 24 hours, the rats were monitored to observe possible immediate effects of vaccination, such as pain, edema, agitation, appetite, sensitivity and behavioral changes. Pain was assessed by observing behaviors indicative of discomfort, such as vocalization and abnormal posture. Edema was checked for the presence of localized swelling in the region where the vaccine was administered. Agitation was analyzed by observing the activity level and restless behavior of the rats. Appetite was monitored to see if there was any change in food intake after vaccine administration. Finally, sensitivity was assessed through gentle tactile stimuli to check whether there was an exaggerated reaction in the rats. After administration of the last dose, the females were maintained until the 21st day of weaning. The data collected were pregnancy rate, fertility rate, live and dead litter and aspects of the litter such as sex, weight and development. The results demonstrated that the administration of the BNT162b2 vaccine had no significant effects on female fertility, showing that all mated females, both in the control and experimental groups, were able to produce a live litter, achieving a copulation rate of 100%, a fertility rate of 100% and a normal pregnancy lasting 21 days. Regarding birth, lactation and postnatal offspring development, the administration of the vaccine had no significant effects on these parameters, generating a total number of normal offspring from 26 females and 29 males from the control group and 21 females and 32 males from the experimental group, with no effects on their physical and functional development. Therefore, it can be concluded that, based on this experimental model, the vaccine is safe to apply before and during the gestational period. The authors would like to thank CNPq and CAPES Scholarship.

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FEMALE REPRODUCTIVE BIOLOGY

Histological evaluation of the female reproductive system of postpartum rats vaccinated with BNT162b2: a vaccine against covid-19

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Due to the pandemic, a lot of work was done to produce efficient vaccines to combat the viral infection for covid-19 and especially their safety to protect the entire population, including pregnant women. However, many studies still need to be carried out to confirm the safety of BNT162b2, especially on the female reproductive system of these pregnant women, since all germ cells are being directly exposed to the vaccine in a period of great susceptibility. Therefore, this study, arising from a comprehensive study, focused on histologically evaluating the possible effects on the female reproductive system of rats vaccinated with BNT162b2 during their reproductive window and pregnancy to ensure the effectiveness of this vaccine during important critical periods of development. The study was approved by the UERN Ethics Committee under protocol number 002/2022. Wistar rats, 18 females and 9 males (for mating) were used. The experimental design was divided into a Control group (n=9 females) treated with saline solution, administered intramuscularly, and an Experimental group (n=9 females) that received the BNT162b2 vaccine at a dosage of 30µg mRNA/dose. The doses were administered 21 days and 14 days before the start of mating and after pregnancy was confirmed on gestational days (DG) 12 and 18, totaling 4 doses. Females were maintained throughout the gestational and postnatal period (PND) until weaning. In this PND 21, the females were euthanized by saturation anesthetic and the ovaries, uterine tube and uterus were collected and processed for histopathological analysis. Histological sections of 5µm were made and stained with hematoxylin and eosin (H&E) to highlight acidic and basic substances, with Mallory's trichrome or Gomori's trichrome to identify collagen fibers (collagen) and observation of the distribution pattern of these fibers. Histological findings indicated that administration of the BNT162b2 vaccine did not result in histological changes in the uterus of females compared to control animals. It was possible to identify the different uterine layers, such as the endometrial mucosa, which is rich in tubular glands and surrounded by fibrous connective tissue (dense, non-modeled connective tissue). The ovaries of vaccinated animals did not show any evident histological changes when observed under optical light microscopy, similar to control group. The medullary and cortical regions are distinct parts of the ovary with different structural characteristics. Both the structures of the cortical layer and those of the medullary layer were preserved and histologically organized. There was an increase in the cortical region of the ovary, characteristic of postpartum. Furthermore, it is possible to observe the presence of several developing follicles and a formed and well-developed corpus luteum. The uterine tube samples were stained with Mallory's trichrome, to visualize collagen fibers in the tissues, in order to verify any pattern of abnormal deposition of these fibers. Again, it was possible to verify that they were normal and without differences when compared to the animals in the control group. In this way, based on this experimental model, it was possible to conclude the effectiveness of the vaccine, at least regarding its safety in the female reproductive system. The authors would like to thank CNPq and CAPES Scholarship.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Bisphenol S chronic exposure interacted with ewe metabolic status to impair female reproduction

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Bisphenol A (BPA), a plasticizer used in the food industry, is reported to be an estrogenomimetic endocrine disruptor, involved in deleterious effects on oocyte meiosis and maturation as well as in steroigegenesis impairment. BPA being regulated, structural analogs emerged including bisphenol S (BPS). Studies on fish and rodent species reported that BPS affects reproduction similarly to BPA. Moreover, because metabolism affects the ovarian functionning, we hypothesized that the metabolic status could interact with the effects of environmental factors. Therefore, this study assessed BPS chronic effects at dose corresponding to the tolerable daily intake defined for the BPA, on oocyte quality, steroidogenesis and granulosa cell proteomic data. Groups of 20 ewes were subjected to either a restricted (R) or well-fed (WF) diet and to a bisphenol daily exposure (0, 4 or 50 µg/kg/day) for more than 3 months, thus generating 6 groups : R0, R4, R50, WF0, WF4 and WF50. After hormonal oestrus synchronization and ovarian stimulation, oocytes were surgically recovered (OPU sessions) and underwent *in vitro* maturation (IVM), fecundation and development. Developmental rates were analyzed at day 2 and 7 after IVM. At the time of slaughter, after 5 month of daily exposure to bisphenol, After hormonal oestrus synchronization, the follicular fluid and granulosa cells of the preovulotary follicle were collected. The follicular fluid underwent a steroidomic analysis while the granulosa cells underwent a proteomic analysis. Body weight was higher in well-fed compared to restricted ewes at the time of oocyte punctures (diet effect, $p<0.0001$, 64.3 ± 1.2 kg vs 54.1 ± 1.2 kg, respectively) which was also the case for body condition score (diet effect, $p<0.0001$, 2.92 ± 0.02 vs 2.18 ± 0.02, respectively). Regarding embryo production data, the most interesting finding was a significant diet x BPS dose interaction that was reported for cleaved embryos, >4-cell embryos, blastocyst and early blastocyst numbers. Moreover, steroidomic analysis of the preovulatory follicle showed a significant interaction between metabolic status and BPS exposure for seven steroids, including estradiol. Indeed, while exposure to BPS impaired estradiol concentrations in follicular fluid of well-fed ewes, this was not reported in restricted ewes. Granulosa cell proteomic analysis of the preovulatory follicle confirmed the interaction between metabolic status and BPS exposure as most of the proteins corresponding to the diet effect (21 and 30 proteins) differ depending on the BPS exposure. Lastly, among the proteins that varied after BPS exposure, the most interesting one is the beta-glucuronidase. Bisphenols, with oestrogenic properties, are metabolized into glucuronide bisphenols, without glucuronide properties. Nevertheless, the beta-glucuronidase has been reported to be able to remove the glucuronide part of BPA glucuronide and therefore to turn it back into BPA with oestrogenic properties. This protein that is expressed at the follicular level, is also overabundant after BPS exposure and could therefore prolonged the BPS effects at the ovarian level. Moreover, according to both the literature and our data, the beta-glucuronidase expression increases with adiposity. To conclude, our data highlighted the deleterious effects of BPS and its interaction with the metabolic status, indicating that its use in food packaging should be regulated. Our data also suggested that individuals with higher adiposity might be more sensitive to bisphenols effects.

Animal Reproduction

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FEMALE REPRODUCTIVE BIOLOGY

EFFECT OF ALPHA-PINENE ON THE ACTIVITY OF ANTIOXIDANT ENZYMES SOD, CAT, GPX AND THIOL LEVELS IN BOVINE OVARIAN TISSUE AFTER VITRIFICATION

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The ovarian tissue cryopreservation (CTO) consists of preserving cells and tissues at low temperatures, aiming to protect the fertility patients undergoing chemotherapy, in addition conserving genetic material from valuable species (1). Although studies present remarkable results, the technique is still greatly affected by oxidative stress resulting from the accumulation of reactive oxygen species (ROS). In this context, different antioxidants have been tested to mitigate the adverse effects of oxidative stress, making the investigation of alpha-pinene (AP), a naturally component with promising potential. Therefore, this study aims to investigate the influence of alpha-pinene at different concentrations (2.5, 25, 10 and 20 µg/mL) on thiol levels and antioxidant enzymes SOD, CAT and GPX, during the process of ovarian tissue vitrification. Firstly, bovine ovaries were collected from a local slaughterhouse, and in the laboratory, the cortex of 10 pairs of ovaries was fragmented and subjected to the vitrification process (2). For this, the fragments were added to the vitrification solution (VS) composed of alpha-minimum essential medium (α-MEM), 10% dimethyl sulfoxide (DMSO), 0.25 mol/L sucrose and different concentrations of alpha-pinene: Group 1: VS without AP (Vitrified control group); Group 2: VS plus 2.5µg/mL of AP; Group 3: VS plus 5µg/mL of AP; Group 4: VS plus 10µg/mL of AP; Group 5: VS plus 20µg/mL. After five minutes in the vitrification solution, the fragments were vitrified and stored in liquid nitrogen. For warming, the samples were immersed in wells containing 1 mL of α-MEM, 10% Fetal Bovine Serum (FBS), and decreasing concentrations of sucrose (0.5M; 0.25M; 0M) for 5 minutes each. They were then macerated in a phosphate buffer and their homogenates centrifuged for spectrophotometric assays, with the data evaluated following the mean ± SEM. The total protein test was performed to obtain the total protein concentration in each sample. The thiol level was determined using 5.5′ -dithiobis(2-nitrobenzoic acid). SOD activity was measured by adrenaline auto-oxidation inhibition. CAT was evaluated by hydrogen peroxide (H2O2) consumption. The enzymatic activity of SOD, CAT, GPX and thiol levels were evaluated by ANOVA and Kruskal-Wallis tests. Regarding SOD activity, a significant increase was observed between the concentration of 2.5µg/m and the α-MEM groups, fresh control, and 20µg/mL, furthermore the concentration of 5µg/mL showed a notable raise compared to the fresh control. GPX levels showed an increase at the concentration of 20µg/mL compared to the fresh control, while thiol levels also increased in the 20µg/mL group compared to the 5µg/mL concentration. The CAT levels were similar at all concentrations. Given the results, alpha pinene treatment can influence the activity of antioxidant enzymes, more specifically on action of SOD at 2.5µg/mL and 5µg/mL concentrations, and GPX elevation at 20µg/mL. Thiol levels did not differ from control fresh, but showed discrepancy between 20µg/mL and 5µg/mL concentrations, demonstrating an increase of levels according to concentration. Then, we can conclude that alpha-pinene added to vitrification solution improved the prooxidant environment through increased thiol levels together with SOD and GPX enzyme activity, making it a viable compound with antioxidant action.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Actaea racemosa (L.) extract (Aplause®) demonstrates protective effects on mice ovarian tissue after chemotherapy induction with doxorubicin

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Doxorubicin (DOX) is an antineoplastic agent used in medical practice. However, there is evidence of its ability to cause damage to the ovary, opening possibilities for studies of plant extracts that potentially have a protective effect on follicular development, such as Actaea racemosa (1,2). Thus, considering the deleterious effects caused by chemotherapy drugs on ovarian function, this study aimed to investigate the potential of A. racemosa extract to reduce ovarian damage caused by DOX in mice ovaries *in vivo*. The Ethical Committee approved the study under number 01/21. The protective effects of A. racemosa extract (0.5 and 5 mg/kg body weight) on mice ovaries that underwent doxorubicin chemotherapy (10 mg/kg body weight) were evaluated. Mice (n = 42) were pre-treated with saline solution (control) or with A. racemosa extract (0.5 or 5 mg/kg) and then were treated after 1h with: (i) saline solution (control); (ii) doxorubicin (10 mg/kg); (iii) 0.5 mg/kg A. racemosa; (iv) doxorubicin and 0.5 mg/kg A. racemosa; (v) 5 mg/kg A. racemosa or (vi) doxorubicin and 5 mg/kg A. racemosa once daily for 10 days. At the end of treatment, the ovaries were collected and fixed for histological analysis to evaluate follicular morphology, development and stromal cell density. Other ovaries were fixed for transmission electron microscopy (TEM). The percentages of primordial, developing follicles and normal follicles, were evaluated by chi-square test. The Kruskal-Wallis test analyzed data of stromal cells, followed by Dunn's comparison. Differences were statistically significant when $p<0.05$. The results show that the administration of 0.5 or 5 mg/kg A. racemosa extract maintained the percentage of morphologically normal follicles, while DOX (10 mg/kg) drastically decreased this percentage. The animals that received both DOX and 0.5 or 5 mg/kg A. racemosa extract had higher percentages of normal follicles than mice that received only DOX. Regarding follicular growth, the DOX (10 mg/kg) reduced the percentage of secondary follicles, but did not influence the percentages of primordial, primary and tertiary follicles. When associated with DOX, the extract of A. racemosa was able to decrease follicular atresia and death in ovarian stromal cells. The ovaries of mice treated with 0.5 mg/kg A. racemosa extract had granulosa cells and oocytes from preantral follicles with well-preserved ultrastructure, besides transzonal projections, homogeneous oocytes with well-defined mitochondria and nuclear membranes. In conclusion, A. racemosa extract maintains follicular survival rates and protects the ovarian follicles and stromal cells against doxorubicin-induced toxicity.

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X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Assessment of stromal cells, granulosa cells survival, and diameter of ovarian follicles included in cultured bovine ovarian tissue

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The ovarian extracellular matrix undergoes remodeling in its structural components that contributes to follicular development. Therefore, characterizing *in vitro* culture-induced changes in ovarian stroma components and granulosa cells of early-stage follicles is a necessary field of research. In this study, we analyzed the effects of *in vitro* culture on stroma cells, granulosa cells, and diameter of ovarian follicles included in bovine ovarian tissue. For this, ovarian fragments were cultured medium consisting of α-MEM supplemented with 1.25 mg/mL of bovine serum albumin, 10 µg/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL selenium, 2mM glutamine, 2mM hypoxanthine, 100 UI/mL penicillin and 100 µg/mL streptomycin (α-MEM+) for 6 days, at 38.5 °C, 5% CO2. Ovarian fragments before (uncultured control) and after culture were fixed for 24 hours at room temperature in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). After fixation, the ovarian fragments were dehydrated in a graded ethanol series, clarified with xylene, and embedded in paraffin wax. For each piece of ovarian cortex, 7 µm sections were mounted on slides and stained with eosin and hematoxylin (H&E) to analyze stromal cell density and measurement of follicular and oocyte diameter. Stromal cell density, granulosa cell number, and follicle and oocyte diameter were evaluated only in morphologically normal primordial and primary follicles after histological processing. Follicles were classified as primordial (one layer of flattened or flattened and cuboidal granulosa cells around the oocyte), primary follicles (one layer of cuboidal granulosa cells around the oocyte). These follicles were classified further individually as histologically normal when an intact oocyte was present, surrounded by granulosa cells that are well organized in one or more layers, and have no pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte, that has a pyknotic nucleus and/or is surrounded by disorganized granulosa cells, which are detached from the basement membrane. Statistical analysis was performed using GraphPad Prism 9 software. Analysis of variance (ANOVA) followed by a Tukey test was applied to compare more than two groups with each other. As a result, it was found that there was a decrease in stromal cell number surrounding the primordial and primary follicles in 6-day cultured tissues compared to the uncultured tissue. Furthermore, when the cultured groups were compared to each other, it was found that the stromal cell number around the primary follicles was lower than in the primordial follicles. Furthermore, after 6 days, primordial and primary follicles showed a significant reduction in the granulosa cells survival and follicular and oocyte diameter. In conclusion, *in vitro* culture of bovine ovarian fragments reduced the density of stromal cells, granulosa cells survival, and follicular and oocyte diameter of primordial and primary follicles. Therefore, there is a necessity to optimize the culture systems, which can be obtained with substance supplementation with growth factors and antioxidants.

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FEMALE REPRODUCTIVE BIOLOGY

EVALUATION OF THE EFFECT OF *In vitro* CULTURE OF THE BOVINE OVARIAN CORTEX ON COLLAGEN DENSITY AND GAGS.

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Ovarian tissue culture technology is a valuable tool for studying the reserve of ovarian follicles. It can enable the production of mature and developmentally competent oocytes by replicating the natural folliculogenesis process in a controlled *in vitro* environment. As cultural systems keep progressing, attention is turning toward early-stage (primordial and primary) follicles, which are plentiful in the ovary, nonetheless, the processes that regulate the activation and development of these follicles remain inadequately understood, and the success of *in vitro* culture has been restricted. Currently, a thorough comprehension of the constituents and roles of the ovarian stroma is a lively field of investigation, holding essential clues to grasp the complex ovarian dynamics. Evidences has shown that the communication between the extracellular matrix (ECM) and follicles, as well as between the ECM and ovarian stromal cells via integrin binding and other receptors, is crucial for supporting the correct process of folliculogenesis. In the ovarian stroma, collagen types 1 and 3 are the predominant matrisome-related proteins. Within this environment, follicles are evenly spread along a finely regulated gradient of collagen within the matrix. The stiffness of the ovaries seems to significantly influence both the resting and active states of follicles. Besides collagen, glycosaminoglycans (GAGs) are heteropolysaccharides, not only involved in the structure of the ECM but also in water retention and growth factor sequestration, being able to promote cell adhesion, growth, differentiation, and migration. Therefore, this study aimed to employ histochemical analyses to investigate the impact of a six-day static *in vitro* culture on collagen density and GAGS. Through this, collagen fibers around primordial and primary follicles in uncultured control and cultured tissue were assessed by staining with Picrosirius Red (Abcam kit), while the preservation of GAGs around primordial and primary follicles was assessed by staining with blue Alcian (pH 2.5). For both analyses, pictures of fifty primordial and fifty primary follicles were captured using a DS Cooled DS DS-Ri1 camera connected to a microscope (Nikon, Eclipse, TS 100, Tokyo, Japan) at 400× magnification. The Fiji-ImageJ software (Version 1.54f, 2023) was employed to measure the percentage of collagen and GAGs by outlining a region of interest (ROI) of 100 µm2 around each follicle. Analysis of collagen and GAGs, key structural components of the ECM, revealed the ECM's preservation status following *in vitro* cultivation. Our findings indicate that *in vitro* cultivation leads to a significant reduction in the proportion of collagen and glycosaminoglycans in ovarian tissue, as evidenced by decreased levels of Picrosirius red solution and Alcian Blue staining, respectively. This reduction impacts the environment surrounding primordial and primary follicles as well as the entire tissue. Furthermore, we observed that the remaining collagen fibers post *in vitro* cultivation exhibited increased curvature, more points of breakage, and a higher number of gaps. These effects could be particularly detrimental to the healthy activation and growth of follicles during *in vitro* cultivation. Analysis of collagen and GAGs, key structural components of the ECM, revealed the ECM's preservation status following *in vitro* cultivation. Our findings indicate that *in vitro* cultivation leads to a significant reduction in the proportion of collagen and glycosaminoglycans in ovarian tissue, as evidenced by decreased levels of Picrosirius Red and Alcian Blue staining, respectively. This reduction impacts the environment surrounding primordial and primary follicles as well as the entire tissue. Furthermore, we observed that the remaining collagen fibers post *in vitro* cultivation exhibited increased curvature, more points of breakage, and a higher number of gaps. These effects could be particularly detrimental to the healthy activation and growth of follicles during *in vitro* cultivation. In summary, based on the findings, it can be concluded that the *in vitro* culture of bovine ovarian tissue leads to the reduction of collagen, glycosaminoglycans, and stromal cell content surrounding the primordial and primary follicles. Thus, we hope that the findings outlined here will inspire efforts to develop more efficient culture systems capable of supporting the *in vitro* healthy growth of ovarian follicles in cattle and other species.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Anethole reduces oxidative stress and improves follicle survival in bovine ovarian tissues cultured *in vitro*

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The *in vitro* culture of preantral ovarian follicles (PAOFs) aims to establish culture systems capable of maintaining follicular survival and development in the early stages to increase the potential of mature and healthy oocytes available for use in techniques such as *in vitro* embryo production (IVEP). One of the obstacles still hindering the advancement of *in vitro* culture of PAOFs from large animals such as cattle is the oxidative stress (OES) caused by high concentrations of reactive oxygen species (ROS) present in the *in vitro* environment. Therefore, it is necessary to use more effective antioxidants to control oxidative stress and thus prevent follicular apoptosis caused by ROS. Natural products have been shown to possess several biological activities, including antioxidant activity. The natural phenylpropanoid anethole has been shown to have antioxidant activity in different culture cell systems. Therefore, this study aims to evaluate the effects of different concentrations of anethole on follicular survival and oxidative stress in bovine ovarian tissues cultured *in vitro*. The activity antioxidant of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), the concentration of thiol and the levels of mRNA for SOD, CAT, GPX1, PRDX6, and NRF2 were also evaluated. To this end, ovaries from 16 cows were collected in local slaughterhouses. In the laboratory, fragments of ovarian cortex (3x3x1 mm) were fixed in 10% paraformaldehyde (uncultured control) or cultured *in vitro* in 500 µL of control medium alone or supplemented with 1, 10, 100 or 1000 µg.mL-1 anethole at 38.5°C with 5% CO2 in the air for 6 days. The control medium was α-MEM supplemented with BSA 1.25 mg.mL-1, glutamine 2 mM, penicillin/streptomycin, hypoxanthine, insulin, selenium 10 µg.mL-1 and transferrin 5.5 µg.mL-1 (α-MEM+). Every other day, 60% the culture medium was replaced. At the end of the culture period, the tissues were fixed in paraformaldehyde 4% and processed for classical histology. The follicles were classified as primordial or developing follicles, as well as on morphologically normal or degenerated. Evaluation of the activity of SOD, CAT and GPX1, concentration of thiol, as well as the levels of mRNA SOD, CAT and GPX1, PRDX6 and NRF2 were performed in tissued cultured in control medium alone or supplemented with anethole 1 µg.mL-1. The percentage of primordial and developing follicles, as well as normal or degenerated follicles were analyzed by Chi-square test ($P < 0.05$). Data of activity on antioxidant enzymes and mRNA expression were analyzed by Kruskal-Wallis test. The results show that tissues cultured in the presence of 1 µg.mL-1 anethole had significantly higher levels of morphologically healthy follicles than those cultured in control medium ($P < 0.05$). In addition, tissues cultured with 1 µg.mL-1 anethole reduced thiol levels. CAT and GPX activity were reduced in tissues cultured in control medium alone or supplemented with anethole. On the other hand, anethole increased CAT activity when compared with control group. The presence of 1 µg.mL-1 anethole reduced the levels of mRNA for CAT, PRDX6 and NRF2 when compared to control medium (α-MEM+) ($P < 0.05$). The data indicate that anethole (1 µg.mL-1) increase follicle survival by regulating oxidative stress, leading to an increase in antioxidant enzyme activities which maintain a favorable redox environment for follicle survival *in vitro*.

Animal Reproduction

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FEMALE REPRODUCTIVE BIOLOGY

ALTRENOGEST SUPPLEMENTATION IN LATE LACTATION IMPROVES UTERINE histoarchitecture without affecting folliculogenesis IN primiparous SOWS

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Altrenogest is a progestagen commonly used for estrus synchronization in pigs. Besides this use, some studies showed that it may improve reproductive performance in primiparous sows. In our previous study, it was reported that altrenogest supplementation in late lactation induced higher progesterone concentrations after estrus, which reduced the incidence of low birthweight piglets. However, the mechanisms that drive the effects on female reproductive function have not been fully elucidated so far. This study aimed to evaluate the effects of ALT supplementation during the last week of lactation on the morphology of female genital tract. Ten hybrid primiparous sows (DB30) were allocated in a randomized design to two treatments: females supplemented with 20 mg of altrenogest orally during the last 6 days of lactation, ending 24 hours before weaning (ALT; n = 5), and non-supplemented sows (CON; n = 5). Sows were euthanized on the first day of estrus and the whole genital tract was recovered. Ovaries and uterus were weighed, whereas the uterine tubes, uterine horns and vaginal lengths were obtained. Diameters of the follicles present on the ovarian surface were determined using a digital caliper. Afterwards, fragments from the right ovary, uterus and uterine tubes were fixed and processed to obtain histological slides. In the ovaries, follicle density (number of follicles/mm²) was determined, as well as the areas of the follicle, antrum, oocyte, and granulosa layer of antral follicles. In the uterine tubes, height and width of folds, height of epithelium and width of connective tissue were measured. In turn, volumetric proportion of endometrial components, glandular density, and average glandular area were obtained in the uterus. Data were analyzed as a randomized complete design and treatment effects were evaluated by the Student-T test. Results were presented as LSMeans ± SEM, and $P \leq 0.05$ was considered significant. No treatment effects were observed on weight and length of the genital tract ($P>0.05$), but uterine tubes of ALT were longer than those of the CON group ($P<0.05$). Altrenogest supplementation did not affect uterine tubes' structure (height and width of folds, height of epithelium and width of connective tissue), follicle density and areas of the antral follicles' components ($P>0.05$). However, higher proportion of endometrial glands and blood vessels, as well as lower proportion of connective tissue were observed in ALT compared to CON sows ($P<0.05$). Mean glandular area was greater in the CON group ($P<0.05$), however higher number of endometrial glands per area were observed in ALT compared to CON females ($P<0.05$). These results strongly suggest positive effects of altrenogest supplementation on the uterine endometrium, leading to hyperplasia of endometrial glands, without affecting folliculogenesis. Such increase in endometrial glands may be related to improvement in histotroph secretion, which is essential for embryonic survival and development. Taken together, the present results indicatet that altrenogest supplementation for six days during the last week of lactation benefit early embryo development.

Animal Reproduction

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FEMALE REPRODUCTIVE BIOLOGY

In vitro culture of sheep secondary follicles in medium supplemented with epigallocatechin-3-gallate

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During *in vitro* culture, ovarian follicles are exposed to oxidative stress due to an increase in the production of reactive oxygen species (ROS), which impairs oocyte quality [1]. An alternative to reducing ROS is the addition of antioxidants to the *in vitro* culture medium, such as epigallocatechin-3-gallate (EGCG) [2], which acts directly on cellular components, eliminating ROS and reducing follicular apoptosis [2,3]. However, the effect of EGCG on the *in vitro* culture of sheep secondary follicles is not yet known. Therefore, the aim of this study was to evaluate its impact on the *in vitro* culture of sheep secondary follicles. Follicles were isolated and cultured at 39°C and 5% CO2 for 18 days in α-MEM+ (control) or this medium supplemented with 0.1, 1 or 10 µg/ml EGCG. After culture, the following endpoints were evaluated: morphology, antrum formation, follicular growth, levels of active mitochondria, glutathione (GSH) and ROS. At the end of culture, no difference was observed (P>0.05) in follicular diameter (average of 335 µm) and antrum formation (average of 83.75%) between treatments. Furthermore, the overall growth rate was similar at concentrations of 1 and 10 µg/ml EGCG compared to α-MEM+. Similarly, following *in vitro* culture of human ovarian tissue, 10 µg/ml EGCG preserved follicular morphology and attenuated cytokine expression caused by doxorubicin treatment [4]. Therefore, it is suggested that 1 and 10 µg/ml of EGCG maintained follicular survival and development by preserving oxidative balance. In addition, EGCG concentrations (1 and 10 µg/ml) did not differ (P>0.05) in terms of mitochondrial activity. However, at a concentration of 0.1 µg/ml of EGCG, there was a significant increase in mitochondrial activity compared to the other concentrations of EGCG and α-MEM+. There was an increase in ROS and a reduction in GSH in treatments with 0.1 and 1 µg/ml of EGCG when compared to α-MEM+ and 10 µg/ml of EGCG, respectively. Considering that GSH protects the follicles by interacting with pro-apoptotic and anti-apoptotic signaling pathways, studies have shown that the reduction of GSH can lead to excessive accumulation of ROS, inducing oxidative stress in the organelles [5]. Therefore, concentrations of 0.1 and 1 µg/ml of EGCG may not have been sufficient to exert its antioxidant capacity. In conclusion, after *in vitro* culture of sheep secondary follicles, the EGCG (10 µg/ml) maintained survival, follicular diameter,antrum formation, general and daily growth, mitochondrial activity, ROS and GSH levels similarly to the control (α-MEM+).

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Mesenchymal Stem Cell Medium in Preantral Follicle Cultures: Effects on Growth and Oxidative Stress

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There are several basic environmental requirements for cells to *in vitro* grow optimally, among this requirement the most important step is selecting an appropriate growth medium for the *in vitro* culture. Thus, the different composition of the media used for preantral (PA) follicles and mesenchymal stem cells (MSC) culture is form the basis for successful *in vitro* growth [1,2]. However, a recent study observed that co-culturing PA with MSC increased the follicular survival and activation, granulosa cell proliferation in relation to the PA treatment alone. These results revealed the remarkable potential of MSC to promote the *in vitro* goat follicular development [1]. However, a question arises: Can the MSC culture medium accommodate and sustain the culture of PA? Therefore, our objective is to investigate how the MSC medium influences follicular and oocyte growth, as well as oxidative stress (reactive oxygen species; ROS levels). This could open new possibilities to optimize co-culture systems and elucidate the cellular interactions between PA vs. MSC. For this purpose, the ovarian cortex (5 animals) was divided into 3 fragments (3×3×1 mm) per animal. For the Uncultured treatment, 1 fragment per animal was immediately fixed and used for histological analysis. The remaining fragments were cultured in 24-well plates with 1 mL of medium per well with PA (commonly used medium for preantral follicles [2] or MSC (commonly used medium for mesenchymal stem cells [1]) treatments. On the days 0 and 7 of culture, the fragments were subject to histological assay and the spent media were subject to assess ROS levels in all the treatments (day 7) was evaluated according to Silva et al (2022) [2]. The variables were analyzed by one-way ANOVA followed by Fisher LSD and Unpaired T-tests. The significance level was set at ($P < 0.05$). After culture, compared to the Uncultured treatment, the MSC treatment reduced ($P < 0.05$) the diameter of only primordial follicles on day 7 of culture. Moreover, the MSC treatment decreased ($P < 0.05$) the diameter of primordial follicles on day 7 compared to the PA treatment. Regarding oocyte diameter, compared to the Uncultured treatment, the PA treatment decreased ($P < 0.05$) the oocyte diameter across all evaluated follicular categories after 7 days of culture. Conversely, the MSC treatment maintained ($P > 0.05$) the diameter of all follicular categories on day 7 of culture. Futhermore, the oocyte diameters of all categories were greater ($P < 0.05$) in the MSC treatment compared to the PA treatment on day 7 of culture. On the other hand, after a 7-day culture period, the MSC treatment resulted in a decrease ($P < 0.05$) in ROS levels in the spent medium compared to the cultured control (PA treatment). The MSC culture medium supported the growth of PA, showing distinct effects on follicular and oocyte dynamics compared to the traditional PA medium. Additionally, lower levels of ROS in the MSC treatment further highlight its potential to create a conducive microenvironment for follicular development. These findings can largely be attributed to the composition of the MSC medium, especially the presence of supplements such as fetal bovine serum (FBS) and non-essential amino acids (NEAA). FBS is recognized for supporting cell growth due to its rich composition of growth factors, proteins, essential nutrients, and natural antioxidants [3]. Previous studies have demonstrated the benefits of FBS in the culture of follicles and oocytes, promoting their survival, growth, and proper maturation [4;5;6]. Moreover, FBS and NEAA present in the MSC medium can serve as precursors to other endogenous antioxidants, such as glutathione, known to combat oxidative stress [7;8]. In conclusion, in addition to promoting follicular growth, the presence of these rich compounds in the MSC medium may have synergistically contributed to the reduction of oxidative stress.

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FEMALE REPRODUCTIVE BIOLOGY

IN VITRO CULTURE OF BOVINE OVARIAN FRAGMENT SUPPLEMENTED WITH ZINC OXIDE NANOCOMPOSITES DOPED WITH CALCIUM OXIDE OR MAGNESIUM OXIDE

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Nanocomposites (NCs) are formed by nanocrystals in the doping process, causing a change in their physical structure, properties and even biological effects, which enhances their functions or reduces undesirable effects, favoring bioavailability and cellular absorption capacity. Therefore, supplementing the culture medium with these NCs doped with Ca2+, Mg2+ and Zn2+ ions can help maintain homeostasis, providing the acquisition of meiotic competence during folliculogenesis and bovine embryonic development, from blastocyst to birth. Thus, the objective was to test the influence of supplementing the *in vitro* culture medium with nanocomposites (NCs) of zinc oxide doped with magnesium chloride (ZnO:0.5 Mg-MgO) and zinc oxide NCs doped with magnesium chloride calcium (ZnO:0.5 Ca-CaO), compared to pure supplementation of these minerals MgCl2 and CaCl2, in three concentrations 10, 20 and 30 µg/ml on the viability of bovine ovarian tissue fragments cultured *in vitro*. For this, 2 experiments were carried out with the following treatments: 1) Fresh Control (CF); Control (CONT); 10 µg/mL calcium chloride (CaCl2) (CA10); 20 µg/mL CaCl2 (CA20); 30 µg/mL of CaCl2 (CA30); 10 µg/mL of ZnO nanoparticles (NP):0.5Ca (NPCA10); 20 µg/mL ZnO NC:0.5Ca (NPCA20); 30 µg/mL ZnO NP:0.5Ca (NPCA30). And 2) CF; CONT; 10 µg/mL magnesium chloride (MgCl2) (MG10); 20 µg/mL of MgCl2 (MG20); 30 µg/mL of MgCl2 (MG30); 10 µg/mL ZnO NP:0.5Mg (NPMG10); 20 µg/mL ZnO NP:0.5Mg (NPMG20); 30 µg/mL ZnO NP:0.5Mg (NPMG30). Tissue degeneration was analyzed using the marker propidium iodide (PI), the respiratory metabolism of cells through the emission of NAD, FAD and redox state, using autofluorescence, production of ROS using dichlorodihydrofluorescein (DCF) and finally, follicular activation and viability, by histological analysis. For statistics, SigmaPlot version 11.0 software (Systat Software, Inc., USA) was used, applying analysis of variance and Fisher LSD post hoc test to compare means, with significance $P < 0.05$. The NPCA20 treatment showed higher levels of cellular degeneration, greater production of ROS and lower emission of NAD(P)H and FAD, compared to CF and its respective CA20 treatment. The NPMG20 treatment showed a lower rate of cell degeneration, greater production of NAD and FAD, lower production of ROS and good follicular viability when compared to controls and MG20. With this we can see that ZnO:0.5Ca NP at a concentration of 20 µg/mL harmed cell and follicular development, proposing cytotoxic effects. In the second experiment, the doping process was efficient in the ZnO:0.5Mg NP, providing a favorable environment for cellular and follicular development. Therefore, the doping process and the correct combination of ions can reverse toxicity.

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FEMALE REPRODUCTIVE BIOLOGY

Effect of polysaccharide extract from the leaves of Cissus sicyoides L. on catalase enzyme activity during *in vitro* culture of goat ovarian tissue

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A major challenge inherent to the *in vitro* culture of ovarian tissue is the occurrence of oxidative stress caused by the excessive production of reactive oxygen species (ROS), such as hydrogen peroxide (H2O2). These species are unstable and highly reactive molecules from mitochondria metabolism during oxygen reduction (1). When produced in physiological concentrations, ROS are important to body functions. However, an imbalance between its production and neutralization by antioxidative agents can result in cellular damage that characterizes oxidative stress (2). In *in vitro* culture of ovarian tissue, oxidative stress can induce follicular atresia. In this context, the use of antioxidant compounds from plants has been a promising alternative for addition to *in vitro* culture media to improve follicular survival and development, as well as promoting oocyte competence. In view of this, the present study aimed to analyze the effect of the polysaccharide extract from Cissus sicyoides leaves on the activity of the catalase enzyme during *in vitro* cult ure of goat ovarian tissue. To this end, fragments of ovarian cortex were cultured for 6 days in 1 mL of medium consisting of α-modification Minimum Essential Medium (α-MEM) supplemented with ITS (10 µg/mL insulin, 5.5 µg/mL transferrin and 5 ng/mL selenium), 2 mM hypoxanthine; 2 mM glutamine; and 1.25 mg/mL of bovine serum albumin, in the absence (cultured control) or presence of different concentrations of Cissus sicyoides polysaccharide extract (20, 40 or 80 µg/mL). The *in vitro* culture was carried out at 39ºC in a humidified atmosphere with 5% CO2, with a total change of the medium every two days. On day zero (D0) and at the end of culture (D6), tissues were collected for biochemical analysis. CAT activity was calculated by consuming H2O2 (hydrogen peroxide) as substrate by spectrophotometry at 240 nm, every 30 seconds for 1 minute. As a result, it was observed that the activity of the CAT enzyme in the groups cultured with 20, 40 or 80 µg/mL of the extract remained equivalent to the both control treatment, fresh control and MEM alone. Maintaining of the enzymatic antioxidant defense system is fundamental for the body, as they work to combat oxidative stress. CAT, for example, plays a central role in the conversion of H2O2 into water and molecular oxygen, preventing the formation of hydroxyl radicals that cause damage to follicular cells (1). Thus, it can be observed that the polysaccharide extract from Cissus sicyoides leaves does not damage the activity of the catalase enzyme during *in vitro* culture of goat ovarian tissue, which implies stating that the extract was not able to enhance the antioxidant activity in the culture system.

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FEMALE REPRODUCTIVE BIOLOGY

The total antioxidant capacity of the ethanolic extract of pomegranate peel (*Punica granatum* L.) and its influence on the survival of *in vitro* cultured bovine pre-antral follicles

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Cattle farming stands out for its significant role in Brazil's agribusiness and economy, as it generates jobs across the country and supplies various animal products to the local, national, and international markets (1). However, there are limitations hindering greater production of animals of this species, such as the conditions under which *in vitro* culture of pre-antral follicles occurs. Among these conditions, variations in light, temperature, and pH favor the overproduction of reactive oxygen species (ROS) and expose cells to oxidative stress damage (2). The aim of the present study was to investigate the antioxidant potential of ethanolic extract produced from pomegranate peel (Punica granatum L.) on bovine ovarian tissue cultured *in vitro*. For extract production, fresh fruits from the local market were collected, and the peels were removed from the fruit and submerged in ethanol (99.8% PA) for 6 days. Finally, the obtained extract was lyophilized, diluted in ultrapure water, and stored at -20°C. The total antioxidant capacity (DPPH and ABTS) of the extract was evaluated. Additionally, ovaries (n=24) from crossbred cows were collected and transported to the laboratory in saline solution with penicillin and streptomycin at 4°C. In the laboratory, ovarian cortexes were fragmented and then cultured for 6 days in αMEM medium added with ITS (10 µg/mL insulin, 5.5 µg/mL transferrin, and 5 ng/mL selenium), BSA (1.25 mg/mL), Glutamine, and Hypoxanthine (αMEM+), in the absence (cultured control) or presence of different concentrations (10, 50, and 100 µg/mL) of ethanolic extract of Punica granatum L. (EE-pPG). The culture was carried out in an incubator at 38.5°C and a humidified atmosphere with 5% CO2. After 6 days, the fragments were subjected to morphological analyses by classical histology. Analysis of variance (ANOVA) with Tukey's or Mann-Whitney post-hoc test, respecting the normality test, was used. A P value <0.05 was considered statistically significant. As results, it was observed that concentrations of 50 and 100 µg/mL of EE-pPG presented significantly higher total antioxidant capacity throughout the culture (D2 and D6) when compared to treatment with 10 µg/mL of EE-pPG. However, only the concentration of 100 µg/mL was able to maintain the percentage of morphologically normal follicles similar to fresh and cultured controls. In this study, we observed a concentration-dependent relationship regarding the total antioxidant capacity of EE-pPG. Possibly, this ability to neutralize free radicals is exerted by phenolic compounds found in pomegranate, mainly punicalagin and ellagic acid. These molecules have around their nuclear structure the presence of hydroxyl functional groups where hydrogen atoms act as potent electron donors, thus exerting antioxidant activity (3). Additionally, the concentration of 100 µg/mL was able to maintain the percentage of normal follicles. Similarly, a previous study described that aqueous pomegranate extract was able to increase the percentage of developing follicles and increase the number of corpora lutea in the ovaries of young and adult rats (4). Therefore, the ethanolic extract of pomegranate peel was able to preserve the morphology of bovine pre-antral follicles, possibly through its antioxidant effect. Among the evaluated concentrations, 100 µg/mL stands out for being able to maintain follicular morphology and prevent oxidative stress.

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FEMALE REPRODUCTIVE BIOLOGY

EFFECTS OF NUTRITIONAL SUPPLEMENTATION OF *CHLORELLA PYRENOIDOSA* ON THE *IN VIVO* DEVELOPMENT OF CAPRINE PREANTRAL OVARIAN FOLLICLES

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Nutrition is considered indispensable for effective and quality reproduction. Furthermore, recent studies have used green microalgae (Chlorella pyrenoidosa) as a source of biomass, due to its nutritional properties (fibers, vitamins, carotenoids) presenting an anti-inflammatory and antioxidant effects [1]. However, the impact of feeding Chlorella pyrenoidosa on the *in vivo* development of goat preantral ovarian follicles (PAOF) remains unknown. Therefore, the aim of this study was to evaluate the *in vivo* development of PAOF from goats after feeding green microalgae (Chlorella pyrenoidosa). Mixed-breed goats (n=14) were selected and divided into two groups: control (n=7), where the animals were fed a standard diet and received 50 mL of water daily by drench for 13 days; and microalgae group (n=7), which animals received a standard diet supplemented by oral administration of 10g of green microalgae (Chlorella pyrenoidosa), dissolved in 50 mL of water using a drench, for 13 days. After slaughter, the pairs of goat ovaries (n = 14) were washed once in 70% alcohol and twice in supplemented MEM-HEPES and transported to the laboratory at 20ºC [2]. In the laboratory, the ovarian cortexes were fragmented and fixed in Davidson's solution for 12 hours. After this period, the ovarian tissue fragments were dehydrated, diaphanized and embedded. The blocks were then sectioned, and the slides stained with periodic acid Schiff (PAS) - hematoxylin. Histological analyses were carried out according to the stage of development of the PAOF (primordial, transition, primary and secondary), and they were classified as morphologically normal or degenerated [3]. A total of 490 preantral follicles were histologically assessed. When follicle survival was assessed between the groups, it was observed that the microalgae group reduced significantly ($P<0.05$) the percentage of morphologically normal follicles compared to the control group. When the categories of normal preantral follicles were compared within each group, the percentage of primordial follicles was statistically higher compared to the other follicles classification in both groups. In addition, the percentage of transitional follicles was significantly higher in the control group compared to the primary and secondary follicles. In contrast, the microalgae group, showed no significant differences in the percentage of developing follicles. These initial results indicate that dietary supplementation with the microalga Chlorella pyrenoidosa may have an impact on the viability and development of preantral follicles.

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FEMALE REPRODUCTIVE BIOLOGY

Ovarian decellularized bioscaffolds increase the viability of bovine preantral follicles cultured *in vitro* in a medium enriched with nanoparticles loaded with resveratrol

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The *in vitro* culture of isolated ovarian follicles to obtain viable oocytes has been investigated by various research groups (1,2). However, after isolation, the follicles lose the natural support structure provided by the adjacent ovarian tissue, which can impair *in vitro* development. Currently, there is growing interest in the use of decellularized extracellular matrix (dECM)-based scaffolds as a niche that can mimic the three-dimensional structure of the ovary for *in vitro* follicle growth (1). In addition, supplementation of the culture medium is a crucial aspect to support follicle survival in culture (2). Recently, resveratrol (Rsv) was associated with reduced apoptosis and enhanced ovarian cell proliferation in various species. However, this compound has limited bioavailability. In this case, encapsulation of Rsv with nanoparticles has been proposed to improve its effect (3). The aim of this study was to develop and to evaluate a protocol for the decellularization of bovine ovarian tissue and to investigate whether this scaffold supports the viability of bovine secondary follicles cultured *in vitro* in a medium supplemented with polymeric NPs containing Rsv (NPRsv). The NPsRsv was performed using the nanoprecipitation technique (4). To obtain the scaffolds (dECM), bovine ovarian cortical fragments were subjected to 3 cycles of freezing and thawing in a -80°C freezer, incubated in 0.1% Triton X-100 solution and 0.5% sodium dodecyl sulfate (SDS), respectively for 9 hours each, at room temperature. Decellularized supports as well as intact fragments (control) were stained with hematoxylin and eosin to confirm cell removal, Picrosirius red and Alcian blue to assess the preservation of collagen and glycosaminoglycans (GAGs), respectively. Fiji-ImageJ software was used to quantify the percentage of collagen and GAGs. Secondary follicles were mechanically isolated from bovine ovaries for *in vitro* culture. Follicles (20/treatment) were cultured for 12 days in two culture systems: (I) 2D system: follicles were cultured in drops of 100 mL of TCM-199+; (II) 3D system: follicles were placed into dECM scaffolds and cultured in TCM-199+ alone (3D control group) or supplemented with 0.02, 0.2 or 2 µM of NPsRsv; 2 µM white NPs (without resveratrol, WhNP); or 2 µM of unencapsulated resveratrol (Rsv). After culture, the follicles were incubated in 4 µM Calcein-AM and immediately imaged with a fluorescence microscope. Fluorescence intensities were quantified using Fiji-Image J software. Fluorescence values of uncultured follicles were chosen as a calibrator to obtain relative fluorescence expression. Quantification of remaining cells, collagen, and GAGs data was compared by the unpaired t-test. Fluorescence intensity was evaluated by ANOVA and Tukey's test ($P < 0.05$). The H&E staining shows that the decellularization method was found to be effective based on the successful removal of cellular components. Staining of collagen and GAGs showed that these ECM components remained intact after decellularization. Follicles cultured in the 3D system exhibit higher fluorescence and this effect is further enhanced by the presence of NPRsv at concentrations of 0.02, 0.2, and 2 µM in the culture medium ($P < 0.05$). When follicles cultured in the presence of 2uM of non-encapsulated Rsv were evaluated, a reduction in follicle viability was observed when compared to those cultured with NPRsv. In conclusion, the decellularized bovine ovarian tissue exhibited characteristics of few reminiscent cells and well-conserved ECM proteins. A decellularized ECM-based scaffold may provide a 3D microenvironment capable of increasing the viability of bovine ovarian follicles cultured *in vitro*. Furthermore, the supplementation of culture medium with resveratrol associated with polymeric nanoparticles enhances the positive effects of 3D ovarian follicles culture in cattle.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

In vitro culture leads to a decrease in enzymatic antioxidant protection in bovine ovarian cortex

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Advances in ovarian tissue culture systems predict the factors that regulate the mechanisms of follicular activation and growth. However, the increase of reactive oxygen species (ROS) plays a critical role in modulating ovarian stromal dynamics, emphasizing the significance of cellular antioxidant capacity during *in vitro* culture, including enzymatic antioxidants (SOD, CAT, GPX, PRDX), as indicators of culture efficiency. Therefore, measuring the changes induced by *in vitro* culture on antioxidant capacity allows the interpretation of current cortical tissue culture systems in domestic species, such as cattle. Thus, this study aims to investigate the changes induced by *in vitro* culture in the mRNA expression for the antioxidant enzymes SOD, CAT, PRDX6 and GPX1, as well as in the activity of SOD, CAT and GPX enzymes, and thiol levels of bovine ovarian tissue. Ovaries from healthy cows, each serving as a replicate (10 replicates, 20 ovaries), were collected with ethical approval, washed, and promptly transported to the laboratory within 1 hour. Fragments from the ovarian tissue were utilized for enzyme activity assessment and mRNA quantification after being cultured in α-MEM (pH 7,2 – 7,4) medium supplemented with 1.25 mg/ml of bovine serum albumin, 10 µg/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml selenium, 2mM glutamine, 2mM hypoxanthine, 100 UI/ml penicillin, and 100 µg/ml streptomycin for 6 days at 38.5 ºC, 5% CO2. mRNA levels of antioxidant genes in ovarian tissue were analyzed using real-time PCR. Total RNA was extracted, and mRNA concentration was assessed. Reverse transcription utilized SYBR Green for quantification. Reactions included specific primers, with GAPDH as the internal control. Reactions were conducted on a Step One Plus instrument, and the 2^-ΔΔCt method was used to analyze the data. Biochemical assays were conducted to assess the enzymatic activity of both uncultured and cultured ovarian tissue fragments (100 mg/ml). The fragments were macerated in a potassium phosphate buffer with protease inhibitors and phenylmethanesulfonyl fluoride and then centrifuged to collect the supernatant for spectrophotometric assays. These assessments included thiol content and the activity of SOD, CAT, and GPx to determine the pro-oxidant status. The total protein concentration was determined using the Bradford method with Coomassie blue, measuring absorbance at 595 nm. A standard curve with bovine albumin (0, 2.5, 5, 10, 15, 25, 35, and 50 mg/ml) standardized pro-oxidant and antioxidant levels. The pro-oxidant activity was measured by thiol content using DTNB, reported as nMol of reduced DTNB per milligram of protein. SOD activity was determined by adrenaline auto-oxidation inhibition, CAT activity by H2O2 consumption, and GPX activity by NADPH oxidation. Data were expressed as mean ± S.E.M. and statistical analysis used GraphPad Prism 9 software. *In vitro* culture of ovarian tissue led to a significant decrease in mRNA levels for CAT, SOD, GPX, and PRDX6 compared to uncultured controls. Additionally, reduced thiol levels and CAT enzymatic activity decreased significantly after 6 days of culture, while no significant differences were observed in SOD and GPX activity between the two treatment groups. In conclusion, increased oxidative stress post *in vitro* culture is evidenced by decreased thiol levels and reduced transcripts of antioxidant enzymes.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Ultra-diluted/dynamized doxorubicin reduces the toxicity caused by doxorubicin during the *in vitro* culture of pig preantral follicles enclosed in ovarian tissue

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Cancer has shown exponential growth over the years, affecting individuals across diverse age groups. In such cases, one of the most recommended treatments is chemotherapy with the administration of doxorubicin (DOX). Despite its efficacy, DOX lacks specificity towards cancer cells, potentially affecting healthy cells, leading to ovarian damage which involves the accumulation of lipofuscin and ovarian fibrosis. Thus, the present study investigated the effect of adding allopathic DOX 0.3µg/ml and the vehicle i.e, 0.2% Ethanol of ultradiluted/dynamized DOX, different dynamizations of ultradiluted/dynamized DOX (6CH, 12CH, and 30CH), both in the absence or presence of chemical stress induced by DOX at 0.3µg/ml on maintenance of type I and III collagen fibers and accumulation of lipofuscin in porcine ovarian tissue cultured *in vitro* for 48 h. In order to achieve this, part of the ovarian tissue fragments was fixed for the Uncultured control and the remaining fragments were cultured in: MEM (cultured control); DOX 0.3µg/ml; Ethanol (0.2%); DOX 6CH; DOX 12CH; DOX 30CH; DOX (0.3µg/ml) + DOX 6CH; DOX (0.3µg/ml) + DOX 12CH; DOX (0.3µg/ml) + DOX 30CH treatments. To evaluate oxidative stress, the lipofuscin staining assay was performed to detect lipofuscin aggresome accumulation (products derived from the peroxidation of lipids and proteins) using the Sudan Black B stain on a subset of the ovarian histological sections. Images obtained were quantified for positive areas stained with lipofuscin using Image J software [1]. The collagen fiber density was evaluated considering the relative areas of fibrosis with rich collagen deposits. Four histological sections per slide were examined using polarized microscopy coupled with an image capture system. The total collagen in the connective tissue and the polarizing colors' differences were analyzed for types I (stained yellow/orange birefringence) and III collagen fibers (stained green birefringence) using Image J software [2,3]. Statistical analysis was conducted using Sigma Plot (version 11.0 Systat Software Inc., USA). Initially, the normality of data distribution (Shapiro-Wilk test) and variance homogeneity (Levene's test) were assessed. All parameters were subjected to One-way ANOVA. A probability of $P < 0.05$ indicated a significant difference. Data were presented as mean (±SEM). Regarding the results, in general, ultradiluted/dynamized DOX mitigated ($P < 0.05$) the toxic effect of allopathic DOX (0.3µg/ml) on the type I and III collagen fibers density, and the production ($P < 0.05$) of lipofuscin in the ovarian tissue. DOX caused damage to the ovarian stroma, in this case, reducing the concentration of collagen type I fibers and stimulating the production of collagen type III fibers. However, the association of allopathic DOX with ultra-diluted/dynamized DOX attenuated damage, bringing it closer to the Uncultured control and similar to the cultured control (MEM). The mitigating effect of dynamized medications regarding stressors has been documented in previous studies [4,5]. In this study, the use of DOX (0.3 µg/mL) resulted in the expected increase ($P < 0.05$) in lipofuscin aggresomes in the cultured tissue. On the other hand, the addition of ultra-diluted/dynamized DOX in association with DOX (0.3 µg/mL) reduced ($P < 0.05$) this intracellular accumulation of lipofuscin aggresomes. This suggests that this action may be associated with a potential reduction in the oxidative stress induced by DOX (0.3 µg/mL), supported by the observed decrease in lipofuscin accumulation in this study. In conclusion, supplementing the culture medium with ultradiluted/dynamized doxorubicin attenuated the toxicity induced by allopathic doxorubicin, reducing the accumulation of lipofuscin and maintaining adequate proportions of type I and III collagen fibers.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

A mathematical model for study of the progesterone curve in Canindé goats

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The understanding the progesterone (P4) curve is important reproductive data as it is related to many reproductive processes, such as length of estrous cycle and consequently the number of follicular growth waves as well as oocyte and embryonic quality after fertilization. The mathematical models are aimed at interpreting how these biological processes work. Thus, the aim of this study was to use a mathematical model that accounts for the variations of P4 during the estrous cycle in Canindé goats in two age groups. Eleven Canindé goats were used, five of which were adults (3-6 years old) and six young (1-2 years old). The estrus was previously synchronized using a hormonal treatment. Five days after estrus, the number of ovulations was determined by ultrasonography (DP-10 Vet Power, Mindray, Shenzhen, China). Blood samples were collected for determination of the plasma P4 concentration. Samples were obtained every 48 h from the onset of estrus until the next estrus or up to 21 days after the first estrus, and the P4 concentrations were measured using a solid-phase radioimmunoassay kit (Coat-A-Count; DPC, Los Angeles, USA). Using the Gauss-Marquardt interactive method, a logistic function was adjusted to model the progesterone curve. All experimental points of the P4 level evolution curve can be represented by a model that considers two trends in the evolution of P4 circulating during the cycle. The onset of estrus (t = 0) corresponds to the instant in which estrus is observed. The first expression of the model is a logistic function and the second is a decreasing exponential function. The following curve parameters were observed: f(0) (progesterone level at synchronized estrus), θ1 (value of the upper limit of the logistic function in nanograms) and Φ (moment of reversal of the phenomenon or time of rupture expressed in days). All calculations for determining the individual curves were performed using the Casio fx-CG50 calculator (Casio, Tokyo, Japan). The values (mean ± SD) between age groups concerning the length of estrous cycle, number of ovulations and P4 parameters were compared by unpaired t test using QuickCalcs software (GraphPad Software, Boston, USA). All females in both groups showed estrus and ovulation after hormonal synchronization treatment. In all parameters studied, without the modeling data, no statistical difference ($P > 0.05$) was observed between age groups. Concerning the mathematical model, two important phases can be distinguished in both age groups: a) a P4 growth phase which begins four days after estrus, and b) a decrease phase which characterizes the involution of the corpus luteum. A higher concentration of P4 was observed in adult goats on days 7 and 9 of the luteogenic phase ($P < 0.05$). Regarding the three parameters studied by the mathematical model, it was observed statistical differences ($P < 0.05$) between age groups. Thus, older females presented higher values for maximum level progesterone after synchronized estrus and for upper limit of the logistic function. Surprisingly, the curve after luteolysis shows a slow downward trend. This result is quite different from similar studies in cattle and European goat breeds. This observation deserves further studies that can elucidate this unexpected behavior in the Canindé breed. The innovation of the model lies in representing the evolution of the P4 level with fewer parameters, chosen for their biological significance. However, exploration of additional parameters could reveal more information about the dynamics of P4 secretion. Acknowledgements: The authors thank the funding agencies FUNCAP (Fortaleza, Brazil) and CNPq (Brasília, Brazil).

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Mangiferin reduces lipofuscin formation in goat ovarian tissue cultured *in vitro*

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The oxidative stress caused by *in vitro* culture negatively affects follicle development due to damage to cell structures and, consequently, can also cause oocyte senescence. Both processes are closely linked to proteostasis disorders caused by protein oxidation and impairment of the proteasomal system, which leads to the accumulation of lipofuscin [1]. As an alternative to this problem, antioxidants such as ascorbic acid have been added to the culture medium. However, it is necessary to use more powerful antioxidants such as mangiferin in the culture medium. Therefore, this study aimed to evaluate the effect of two concentrations of mangiferin (10 µM- MANGI 10 and 50 µM- MANGI 50) on the production of lipofuscin in goat ovarian tissue and to compare its effect with that of ascorbic acid (50 µg/mL- AA) [2]. To this end, three pairs of ovaries were collected, fragmented, and randomly divided into the following treatments: uncultured control, MEM (cultured control), AA, MANGI 10, and MANGI 50. The cultivation was carried out for 7 days (38.5ºC, 5% CO2 in air), with the medium changed every two days. To evaluate oxidative stress, the lipofuscin staining assay was performed to detect lipofuscin aggresome accumulation (products derived from the peroxidation of lipids and proteins) using the Sudan Black B stain on a subset of the ovarian histological sections. Images obtained were quantified for positive areas stained with lipofuscin using Image J software [3]. All parameters were subjected to One-way ANOVA. A probability of $p < 0.05$ indicated a significant difference. Data were presented as mean (±SEM). Regarding the results, it was observed that compared to the fresh control, only MANGI 50 maintained lipofuscin production rates, while the other treatments reduced (MEM) or increased (AA and MANGI 10) ($p<0.05$) this production. About MEM, all the treatments significantly increased ($p<0.05$) lipofuscin production. However, the MANGI 50 treatment had the lowest increase compared to the other treatments. Lipofuscin is present in most cell types however, studies show that increased accumulation of lipofuscin is directly related to oxidative stress [4], which suggests that the treatments that showed increased lipofuscin production were more subject to the effects of oxidative stress. The MANGI 50 treatment was the only one to maintain lipofuscin rates similar to the fresh control, given that, thanks to its heterocyclic ring structure, it facilitates the elimination of reactive oxygen species (ROS), consequently reducing oxidative stress and the accumulation of lipofuscin [5]. Although ascorbic acid is a well-established antioxidant in the literature, the antioxidant potential of mangiferin is higher than that of ascorbic acid [6]. Thus, it can be concluded that mangiferin at a concentration of 50 µM reduces the production of lipofuscin in the *in vitro* culture of goat ovarian tissue.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Extracellular vesicles from bovine follicular fluid can be incorporated with a scrambled miRNA without affecting their uptake by bovine cumulus cells cultured *in vitro*

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During follicular and oocyte development, the transfer of biomolecules between somatic cells and the gametes is crucial for oocyte quality1. This transfer is facilitated by follicular fluid, which provides a vital microenvironment for enabling this communication between somatic cells and the oocyte2. Among its components are extracellular vesicles (EVs), which carry various biological molecules including miRNAs capable of modulating the function of recipient cells3. Therefore, the hypothesis of this study is that extracellular vesicles from follicular fluid (FF) may incorporate a scrambled miRNA and deliver it to cumulus cells *in vitro*. For this purpose, bovine ovarian FF -derived extracellular vesicles (EVs) were isolated using size exclusion chromatography (SEC), and their characterization was conducted using nano-flow cytometry, transmission electron microscopy (TEM), and nanoparticle tracking analysis (NTA). Subsequently, scrambled miRNA molecules labeled with a fluorophore (Alexa 488) were incorporated into EVs using the Exosome Transfection Kit (Exo-fectTM). The validation of incorporation was also performed using nano-flow cytometry, TEM, and NTA. Following this a cell line derived from bovine cumulus cells was cultured, and upon reaching the fourth passage, the cells were divided into the following experimental groups: (1) negative control (comprising only cumulus cells); (2) a group supplemented with isolated EVs from FF; (3) a group treated with EVs and Exo-fectTM; (4) a group treated with EVs and scrambled miRNA ; (5) a group treated with Exo-fectTM and scrambled miRNA ; and (6) a group treated with incorporated EVs (EVs + Exo-fectTM + scrambled miRNA). The supplementation lasted for 2 h, after which endocytosis was evaluated using an inverted microscope (Thunder Imager 3D Assay Leica Microsystems, Wetzlar, Germany). The techniques used for EV characterization revealed cup-shaped structures with a size and concentration of 152 nm and 1.91 x 1011 particles/mL, respectively, in addition to positive signals for EV markers (CD81, Alix, Syntenin, and Calcein). Post-incorporation, there was no significant difference observed in morphology, size, and concentration between the FF EVs and the incorporated EVs, according to the Tukey test ($P \leq 0.05$). Nano-flow cytometry was performed (n=3) to identify the number of positive events per µL for the fluorophore conjugated with the scrambled miRNA incorporated into the EVs, which exhibited a notably higher count in the group of incorporated EVs compared to the control groups, as confirmed by the Tukey test ($P \leq 0.05$). Taken together, these findings provide compelling evidence that the incorporation was executed efficiently without inducing alterations in the morphology and size of the EVs. Examination of endocytosis by cumulus cells revealed that cells supplemented with EVs subjected to the scrambled miRNA incorporation protocol showed fluorescent punctate structures, both intracytoplasmic and perinuclear, suggesting the occurrence of endocytosis. Furthermore, the fluorescence intensity (n=4) observed in this group was statistically different from that of the control groups ($P \leq 0.05$). Thus, it is evident that the presence of EVs as a natural delivery mechanism for cells is indispensable, and synthetic incorporation of a bioactive molecule does not compromise endocytosis by cumulus cells cultured *in vitro*.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

The morphological classification of feline oocytes significantly influences their vitrification outcomes

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Oocyte vitrification is a technique that allows the preservation of female genetic material and may be fundamental to the conservation of endangered wild felid species. The domestic cat is commonly used as a model for those wild felids, as it is well-known that their genetic material is extremely valuable. After retrieval from the follicles, the oocytes are usually found as cumulus-oocyte complexes (COCs) within variable numbers of cumulus cell layers, and a grade classification based on that and ooplasm morphology is often used worldwide. Even though typically only grade I COCs are used for vitrification, assessing the feasibility of grade II or III may be important for biodiversity preservation. This study evaluated the viability of using oocytes presenting different grades regarding their morphological quality for vitrification. For this, cat ovaries obtained from elective sterilization procedures at local veterinary clinics were submitted to a slicing procedure, and the recovered COCs were morphologically classified and allocated according to the experimental groups: Grade I (G1; uniform and dark cytoplasm containing five or more layers of cumulus cells), Grade II (G2; uniform and dark cytoplasm containing <5 layers of cumulus cells), and Grade III (G3; heterogeneous cytoplasm; partially or completely denuded). For vitrification, pools of 5 COCs were incubated for 15 min in TCM199 supplemented with 7.5% ethylene glycol (EG), 7.5% dimethyl sulfoxide (DMSO), and 20% fetal bovine serum (FBS) on the heating plate. Then, COCs were incubated in TCM199 supplemented with 15% EG, 15% DMSO, 0.5 M sucrose, and 20% FBS (vitrification solution), placed on a cryotop, and immersed in liquid nitrogen within 90 s. For warming, the COCs were incubated in TCM199 medium with 20% FBS containing decreasing concentrations of sucrose (1 M for 1 min, 0.5 M for 3 min, and 0 M for 5 min) at 37° C. Post-warming COCs were analyzed for oocyte viability [Neutral Red (NR)], nuclear oocyte maturation (Hoechst), mitochondrial activity (Mitotracker Red), glutathione (Cell Tracker Blue CMF2HC) and reactive oxygen species [(ROS); H2DCFDA] levels. The variables were tested for normality (Kolmogorov-Smirnov test) and homoscedasticity (Bartlett's test), and subsequently, they were evaluated by ANOVA followed by the Tukey test. The oocyte viability rate and the nuclear maturation were assessed using the chi-square test. The G1 oocytes showed lower ($P<0.05$) levels of ROS (G1: 28.2 ± 1.7 vs G2: 53.4 ± 2.5 vs G3: 53.2 ± 5.0 AU) and mitochondrial activity (G1: 78.2 ± 4.7 vs G2: 140.3 ± 4.6 vs G3: 160.5 ± 10.1 AU) when compared to G2 and G3. Interestingly, GSH levels and viability rate were significantly higher ($P<0.05$) in G2 (GSH: 5.3 ± 0.2 AU and viability: 88.2%) compared to G3 (GSH: 4.0 ± 0.4 AU and viability: 37.5%), while G1 was intermediary and similar ($P>0.05$) to both other groups (GSH: 4.7 ± 0.1 AU and viability: 70.6%). Those differences in the metabolism were not able to affect the oocyte nuclear maturation rate, which was similar ($P>0.05$) among all treatments. The metaphasis II rate was 7% (2/28) for G1, 17% (4/23) for G2, and 13% (3/23) for G3. In conclusion, the morphological classification of COCs impacts several parameters related to the competence of vitrified-warmed feline oocytes. Although G3 oocytes have lower viability and antioxidant defenses, they can still be used to safeguard the genetic material of genetically valuable females. Financial support: CAPES (code 001), CNPq, and FAPERJ.

Keywords: biodiversity; cat; cryopreservation; metabolism.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Litter histological evaluation from rats exposed to BNT162b2: a vaccine against covid-19

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The rapid spread of the SARS-CoV-2 virus has generated unprecedented demand for the development of vaccines capable of preventing infection and the disease' spread. Among the vaccines developed, BNT162b2, a messenger RNA (mRNA) vaccine, has stood out for its effectiveness in preventing Sars-Cov-2 infection and for its indication by government agencies for use in pregnant women. However, little is known about the possible effects of this exposure during a critical window of development. In this context, this study, arising from a comprehensive study, focused on analyzing the embryo-fetal and histological development of fetuses from animals exposed to BNT162b2 before and during pregnancy. The study was approved by the UERN Ethics Committee under protocol number 002/2022. Wistar rats, 18 females and 9 males (for mating) were used. The experimental design was divided into a Control group (n=9 females) treated with saline solution, administered intramuscularly, and an Experimental group (n=9 females) that received the BNT162b2 vaccine at a dosage of 30µg mRNA/dose. The doses were administered 21 days and 14 days before the start of mating and after pregnancy was confirmed on gestational days (GD) 12 and 18, totaling 4 doses. Females were maintained throughout the gestational period and at GD20, the females were euthanized by saturation anesthetic and the pregnant uterus was removed and weighed, the fetuses were removed from the uterus and weighed individually. A detailed external examination of each fetus occurred, which included assessment of palatal closure and sex determination. External findings were observed to analyze the occurrence of developmental abnormalities, variations or malformations. Photomicrographs were performed using a stereoscope in order to evaluate possible changes present in these fetuses after treatment. However, no obvious changes were observed, such as malformation, absence of any structure or congenital anomalies in fetuses from vaccinated animals when compared to the control group. To better characterize these results, photomicrographs of the histological slides of the fetuses were taken under an optical microscope and stained with different dyes. Some structures were observed that have a predisposition to present some type of malformation during embryonic development. Among the structures observed, we can note that the fetal eyes have all layers and structures intact. Furthermore, it was possible to observe that all striated skeletal muscles were fully developed in different regions of the body. The bone structures were also intact and in full development and this was easily proven when observing the endochondral ossification processes in broad development. Another very important point concerns the development of organs. When we evaluated the development of specific organs, no apparent microscopic changes were also observed. An evaluation of essential organs such as the liver was carried out and we noticed that the entire parenchyma of the organ was preserved and full of hepatocytes, as expected. Regarding the kidneys, emphasis is placed on the renal glomeruli in full final development, with correct positioning in the cortical region of the organ, with extensive development of the renal tubules (proximal and distal convoluted tubules), which characterizes a normal development of the organ, based on its basic functional structure, which is the nephron. Another organ evaluated was the lung and we noticed that, like the others, all its structures were preserved, especially the pulmonary bronchioles easily observed in several regions evaluated. Although many organs were analyzed, we did not find any morphological changes, which range from the anatomical to the microscopic part (histological organization) when comparing the experimental group with the control. Therefore, we can conclude that no evidence of changes was found in the tests carried out, which further proves the safety and use of the BNT162b2 vaccine during pregnancy.

Animal Reproduction

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FEMALE REPRODUCTIVE BIOLOGY

Gene expression pattern in the different grades of *in vitro* matured oocytes and their *cumulus* cells under the same microenvironmental conditions in bubaline and bovine

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Assisted reproductive technologies used in cattle are also adopted in buffalo species. Today, it is well known that the physiology and reproductive behavior of buffalo differs from that of cattle. Therefore, comparative gene expression studies in oocytes and cumulus cells offer insights into similarities and differences in molecular mechanisms influencing oocyte quality and cumulus cell function. Thus, this study investigated the gene expression patterns in different grades of *in vitro* matured oocytes and their cumulus cells in bubaline and bovine under the same microenvironmental conditions in culture. Ovaries from both species were collected in abattoir and promptly transported to the laboratory. The cumulus-oocyte complexes (COCs) were aspirated and divided into two groups: group A (grade I and II) and group B (grade III). Subsequently, the COCs were subjected to *in vitro* maturation (IVM) for 24 hours. Right after, the cumulus cells were separated from oocytes using hyaluronidase and protease. The separated oocytes (10 per pool) and their respective cumulus cells were stored at -80 °C until further use. Real-time PCR was performed to verify gene expression in oocytes for CX43 (maturation), ZP3 (fertilization), BCL2 (anti-apoptotic), and TFAM (mtDNA replication) and their cumulus cells for FSH, BCL2 (regulator of apoptosis), and TFAM (mitochondrial activity) of different groups. The gene expression did not differ significantly in Group A and Group B oocytes and their cumulus cells for both the species except in bovine, where the BCL2 was more expressed ($P < 0.05$) in the Group A oocytes than in Group B. The gene expression pattern showed that the molecular communications, fertilizing capacity, antiapoptotic mechanism and mitochondrial activities were similar for both groups in both species. In bovine, the BCL2 gene was more expressed ($P < 0.05$) in the Group A oocytes indicating a better antiapoptotic mechanism than Group B. When comparing the two species, the BCL2 and TFAM were significantly ($P < 0.05$) more expressed in bovine species than bubaline in Group A oocytes. For Group B oocytes, the COX43 gene was significantly ($P < 0.05$) more expressed in bubaline when compared to bovine. The expression of FSH, BCL2 and TFAM in cumulus cells of the Group A oocytes were more expressed ($P<0.05$) in bubaline as compared to bovine. For the cumulus cells of Group B oocytes, the expression of FSH and BCL2 was significantly ($P<0.05$) higher in bubaline when compared to bovine. Interestingly, when comparing the two species, the level of gene expression varied in both oocytes and cumulus cells, suggesting species-specific differences in their regulation. In conclusion, the study suggests that the expression of CX43, ZP3, BCL2, and TFAM genes may not be significantly associated with the differentiation of oocyte grades in bubaline species. However, BCL2 may play a role in the differentiation of oocyte grades in bovine species. Furthermore, there are species-specific differences in the expression of these genes, indicating their potential role in species-specific oocyte development and cumulus cell function. Further research is needed to fully understand the functional significance of these gene expressions in both species. Acknowledgements: Fazenda Laguna (Paracuru, Brazil) and FUNCAP (Fortaleza, Brazil, grant # DEP-0164-00341.01.00/19).

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FEMALE REPRODUCTIVE BIOLOGY

ASSESSMENT OF THE TOXICITY OF CHROMOMYCIN A5 AND THE PROTECTIVE EFFECT OF ALA ON BOVINE OOCYTES

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Chromomycin A5 (CA5) is a metabolite isolated from marine bacteria of the genus Streptomyces that has been extensively studied by several researchers due to its cytotoxic potential on different cancer cell lines [1]. However, its mechanism of action can be toxic to healthy cells, including cytotoxicity in gametes, similar to doxorubicin (DXR). Thus, one strategy to reduce the toxic effects of chemotherapy on gametes, specifically oocytes, is to combine chemotherapy with antioxidants, such as alpha lipoic acid (ALA). Therefore, the aim of this study was to evaluate the effects of CA5 alone or in association with ALA added to the *in vitro* maturation (IVM) medium of bovine oocytes on oocyte viability, chromatin configuration and intracellular production of reactive oxygen species (ROS). For this purpose, cumulus-oocyte complexes (COC) were matured *in vitro* in TCM-199+ medium in the absence (control) or presence of different concentrations of CA5 (50, 80, 100 and 200 nM), in order to define the concentration of LD50. Subsequently, COC were matured *in vitro* in TCM-199+ medium (control) or TCM-199+ supplemented with: 100 nM doxorubicin (DXR100); 200 nM CA5 (CA5200); 100 µM ALA (ALA100); or 200 nM CA5 and 100 µM ALA (CA5+ALA). After IVM, the COC were denuded and the oocytes were analyzed for viability and chromatin configuration, as well as ROS intracellular levels. The data related to the definition of the LD50 concentration of CA5 showed that 200 nM of CA5 resulted in the lowest rates of oocytes in metaphase II (MII) ($P<0.05$) compared to the control. The ALA and CA5+ALA treatments had higher rates of oocytes in metaphase stage I compared to the control. On the other hand, the DXR, CA5 and CA5+ALA treatments had lower rates of oocytes in MII than the control ($P<0.05$). In addition, the CA5 and CA5+ALA treatments showed lower rates of MII than the oocytes exposed to ALA alone. Similar results were observed for the rate of oocyte degeneration, both in relation to ALA and the control. In relation to ROS levels, the ALA and CA5+ALA treatments were lower than the control ($P<0.05$). In addition, the levels of ROS in the ALA treatment were lower than those observed in the presence of DXR. According to the results presented, it is known that CA5 breaks the DNA double strand and consequently causes an increase in the expression of genes related to apoptosis [2]. Therefore, we suggest that this mechanism was probably responsible for the deleterious effects of CA5 on oocytes, such as low MII rates, high degeneration rates and increased levels of ROS. In addition, the beneficial effect of ALA on oocytes was demonstrated. This is due to ALA's ability to stabilize reactive species and stimulate endogenous cellular antioxidant activity [3]. In conclusion, our results revealed that CA5 negatively affected *in vitro* maturation and increased bovine oocyte degeneration, similar to DXR. Furthermore, although ALA at the concentration tested was not able to attenuate the damage caused by this metabolite, it did have a positive effect on ROS levels.

Keywords: Cromomycin. Chemotherapy. Bovine oocytes. Oocyte maturation. Alpha-lipoic ácid.

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FEMALE REPRODUCTIVE BIOLOGY

EVALUATION OF OVARIAN TOXICITY OF WITHANOLIDES DERIVATIVES IN HEALTHY MICE

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Female infertility is a major concern for women of reproductive age undergoing antineoplastic therapy [1], as it can lead to depletion of ovarian follicular reserve, resulting in premature ovarian failure (POF) [2]. Therefore, significant efforts have been made to identify compounds with low adverse effects on the ovarian follicle population, particularly preantral follicles, among chemotherapy agents. In this context, withanolides derivatives such as Withaferin A (WTA), 27-deoxy-24,25-epoxywithaferin A (WT1), and 27-deoxywithaferin A (WT2) emerge as a class of secondary metabolites known for their high chemotherapeutic potential [3] and low toxicity in healthy cells [4]. However, their effects on the ovary remain unknown. Thus, the objective of this study was to evaluate the *in vivo* effect of withanolides derivatives (WTA, WT1, and WT2) on preantral ovarian follicles in mice. To achieve this, 35 female C57BL6J mice were randomly assigned to 7 experimental conditions (n=5/treatment), receiving either 7 doses of saline solution (control treatment) or 7 doses of withanolides derivatives (WTA, WT1, and WT2) at concentrations of 5 or 10 mg/Kg via intraperitoneal injection every 48 hours for 15 days. Forty-eight hours after the last dose (day 13), the females were euthanized by an overdose of ketamine/xylazine solution and cervical dislocation. Subsequently, the pairs of ovaries were immediately collected and processed for evaluation of immunolabeling of DNA damage proteins (γH2AX) and apoptosis (activated Caspase-3). Statistical analysis was carried out using Sigma Plot version 11.0. Comparison of means were analyzed by one-way ANOVA followed by the Fishers least-significant difference (LSD) test. The proportion variables were compared among treatments by chi-square or Fisher Exact tests. Data are presented as mean ± (SEM) and percentage and the results were considered different when $P<0.05$. Regarding γH2AX, the percentage of positive cells at the concentration of 10 mg/Kg was higher ($P<0.05$) compared to the concentration of 5 mg/Kg for all withanolides derivatives (WTA, WT1, and WT2). Additionally, the percentage of marked cells in the WT2 treatment at 10 mg/Kg was higher than the control treatment ($P<0.05$). As for labeling for activated Caspase-3, the percentage of positive cells did not differ between the control treatment and withanolides derivatives at the concentration of 5 mg/Kg ($P>0.05$). On the other hand, in the WT1 treatment at 10 mg/Kg, labeling for activated Caspase-3 was higher compared to the control ($P<0.05$). Moreover, labeling for activated Caspase-3 was higher at 10 mg/Kg of WTA compared to the concentration of 5 mg/Kg of the same drug ($P<0.05$). Based on the presented results, we can suggest that the use of withanolides derivatives (WTA, WT1, and WT2) at a concentration of 5 mg/Kg does not cause sufficient DNA damage to provide a detectable degree of γH2AX in mice follicles, as evidenced by the lack of difference in labeling for activated Caspase-3. On the other hand, we conclude that in higher concentrations, such as 10 mg/Kg, withanolides derivatives cause DNA damage affecting the integrity of mice preantral follicles.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Influence of the essential oil of *Croton Argyrophyllus* Kunth on follicular activation and extracellular matrix remodeling during *in vitro* culture of bovine ovarian tissue

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The *in vitro* culture of ovarian tissue has allowed advances in knwoledges related to ovarian follicles activation and development of preantral follicles *in vitro*. Advances in this essay can improve the rates of animal production by reproductive biotechniques. However, the use of the ovarian follicular reserve still faces challenges, mainly due to oxidative stress. The present study aimed to evaluate the effect of C. argyrophyllus Kunth essential oil (CAEO) on *in vitro* follicular activation and extracellular matrix integrity after six days of *in vitro* culture of preantral follicles enclosed in bovine ovarian tissue. In the laboratory, ovarian cortex fragments (3x3x1 mm) were fixed in 10% paraformaldehyde (uncultivated control) or cultured *in vitro* in 500 µL of alone control medium (α-MEM⁺) constituted with BSA (1.25 mg/mL), glutamine (2 mM), penicillin/streptomycin (100 µg/ml), hypoxanthine (2 mM), insulin (10 µg/ml), selenium (10 µg/mL), and transferrin (5.5 µl/mL) or supplemented with different concentrations of EO from C. argyrophyllus Kunth (0.01, 0,1, 1, 10 and 100µg/mL) at 38.5°C, in 5% CO2 in air for 6 days. At the end of the culture period, the tissues were fixed and processed for classical histology (Hematoxylin & Eosin) to evaluate activation and the distribution of collagen fibers in the extracellular matrix (picrosirius red). Statistical analysis was performed using GraphPad Prism (9.0) software. Follicular activation in each treatment was assessed by Tukey's test, and comparison between treatments was performed by Fisher's exact test. Collagen fiber distribution data were analyzed by Kruskal-Wallis test, followed by Dunn's comparison. Differences were considered statistically significant when $P < 0.05$. The results showed a significant reduction in primordial follicles in the groups treated with 0.01, 0.1, and 100 µg/mL of CAEO when compared to the cultured control and the uncultivated control ($P < 0,05$). In the analysis of the extracellular matrix, the treatments at different concentrations of CAEO did not show a difference compared to the cultivated control, but they differed in the MEM, 0.01, and 100 µg/mL groups when compared to the uncultivated control, exhibiting a reduction in collagen fibers. Based on the analyses conducted in this study, it is concluded that the EO of C. argyrophyllus Kunth, at concentrations of 0.01, 0.1, and 100 µg/mL, allowed follicular activation. However, the treatments with different concentrations of CAEO did not differ from the cultivated control regarding collagen distribution in the extracellular matrix.

Keywords: Culture *in vitro*. Coton Argyrophyllus Kunth. Extracellular matrix. Follicular activation.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Effect of lactose on the *in vitro* development of sheep secondary follicles

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In vitro culture of preantral follicles is a promising biotechnology for increasing the quantity of meiotically competent oocytes for embryo production (1). However, due to suboptimal *in vitro* culture conditions, the production of viable oocytes remains unsatisfactory in ruminants (2). Therefore, supplementing the culture medium with energetic substrates, essential for follicular development and the regulation of oocyte metabolism and viability (3), such as the disaccharide lactose (galactose β-1,4 glucose), may offer a viable alternative for increase oocyte production from *in vitro* culture of preantral follicles (3). The aim of this study was to evaluate the effect of lactose on the morphology, development, glutathione (GSH) and mitochondrial activity levels, DNA fragmentation, and meiotic resumption of oocytes from sheep secondary follicles cultured *in vitro*. Isolated secondary follicles were cultured individually for 18 days in alfa-modified minimal essential medium (α-MEM) supplemented with 3.0 mg/ml bovine serum albumin (BSA), 10 ng/ml insulin, 2 mM glutamine, 2 mM hypoxanthine, 5.5 µg/ml transferrin, 5.0 ng/ml selenium and 50 µg/ml ascorbic acid (control medium: α-MEM+) or in α-MEM+ with different concentrations of lactose (0.025, 0.05 and 0.1 M). After culture, some of the oocytes underwent TUNEL assay and *in vitro* maturation (IVM). On day 18, the percentage of morphologically normal follicles (92.5%), the levels of GSH and active mitochondria, and the rate of meiotic resumption increased ($P<0.05$) in the treatment with 0.025 M lactose compared to the control group (75.55% normal follicles). Furthermore, the control group (35.09%) showed a higher percentage of TUNEL-positive oocytes compared to 0.025 M lactose (9.09%). Based on these results, it is possible to suggest that lactose (0.025 M), through its metabolites (glucose and subsequent production of pyruvate and lactate), protected oocytes against oxidative stress *in vitro* by increasing the production of GSH and mitochondrial activity, enhancing follicular survival, and inhibiting DNA fragmentation. This hypothesis is based on previous studies that mention the hydrolysis of lactose into glucose and galactose, catalyzed by enzymes called β-galactosidases (4), which are present in ovarian follicular fluid (5). Additionally, these studies indicated that glucose increased proliferation and steroid production in ovine granulosa cells cultured *in vitro* (6). In conclusion, 0.025 M lactose increased the survival, GSH and active mitochondria levels, and meiotic resumption of oocytes from *in vitro* cultured secondary follicles.

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Animal Reproduction

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FEMALE REPRODUCTIVE BIOLOGY

Impact of the addition of Anethole or Alpha-Lipoic Acid during the *in vitro* culture of vitrified caprine secondary and early antral ovarian follicles

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Cryopreservation of isolated ovarian follicles is a promising tool for preserving female fertility (1). During the *in vitro* culture of these follicles, several factors can cause an imbalance in the antioxidant defense system, culminating in excessive accumulation of reactive oxygen species (ROS), which can have deleterious effects, reducing follicular quality (2). Therefore, this study aimed to compare the use of two antioxidants, Anethole (AN) or Alpha-Lipoic Acid (ALA), in the *in vitro* culture of vitrified and fresh goat ovarian follicles. Secondary (SEC) (n=160) and early antral follicles (EANT) (n=160) were isolated and randomly allocated into two groups: fresh or vitrified. Fresh or vitrified SEC and EANT were culture in a medium supplemented with ALA at 100 µM (ALA100) or AN at 300 µg/mL (AN300) for 12 days. During and at the end of *in vitro* culture, the morphology, follicular development (antrum formation and growth rate), and hormonal levels were analyzed. The data revealed that the percentage of morphologically normal follicles was similar between fresh and vitrified EANT follicles, regardless of the antioxidant used ($P<0.05$). In addition, the morphology of vitrified SEC follicles was not affected, regardless of the antioxidant used ($P<0.05$). The antrum formation rate was higher in the fresh AN300 group compared to the fresh ALA100 group ($P<0.05$). The growth rate in the group fresh AN300 was higher ($P<0.05$) compared to fresh ALA100 and was similar before vitrified AN300 in both follicular categories. However, vitrified follicles cultured with ALA100 showed a negative growth rate. At the and culture, the estradiol production was higher in group fresh SEC AN300 compared to fresh SEC ALA100 and similar to vitrified SEC AN300 ($P<0.05$). In addition, there was an increase in hormonal production in the vitrified SEC follicles ($P<0.05$). However, in the EANT follicles, the estradiol production in the vitrified AN300 group was similar to that in the fresh AN300 group, which was higher than in the vitrified ALA100 group. In conclusion, addition of antioxidants (ALA and AN) during *in vitro* culture did not affect the morphology of fresh or vitrified ovarian follicles. However, the seems to be more efficient in promoting follicle development and growth. Further studies are therefore recommended to evaluate the follicle development over a longer period (18 days) to obtain potentially fertilizable oocytes for IVP.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Phenazine ethosulfate did not reduce the reactive oxygen species (ROS) and the lipid content in swine embryos produced *in vitro*

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The high lipid content and oxidative stress are limiting factors for the development of swine embryos *in vitro*. Previous studies have reported that phenazine ethosulfate (PES), an electron acceptor associated with NADP(H) regeneration, can reduce the lipid content in swine embryos cultured *in vitro* after cleavage. Based on this, we hypothesize that distinct concentrations of PES (0.05; 0.1 and 0.2 µM) during the initial *in vitro* culture of swine embryos could reduce their lipid and ROS content. Sow ovaries were collected in an abattoir and oocytes were obtained through follicle aspiration, followed by selection, maturation, and parthenogenetic activation. Embryos were cultured in PZM-5 medium for 7 days (until D7) with feeding (10% FCS supplementation) at D5. The effect of adding PES at distinct concentrations for 48h was evaluated in four treatments: control (no PES); T1 (0.05 µM); T2 (0.1 µM); and T3 (0.2 µM). Five replicates were conducted each with 20-25 embryos per group. Cleavage and blastocyst development rates were assessed on D2 and D7, respectively. Three to five structures (on D2) and at least six embryos (on D7) per treatment were selected and stained for 30 minutes either with 10 µm 2', 7'-dichlorofluorescein (DCFH) for ROS levels, or with 20ug/ml Bodipy 493/503 for lipid content. All structures were also stained with 10ug/ml Hoechst 33342 for blastomere count. An epifluorescence microscope was used for evaluation with images taken immediately, and the fluorescence intensities in the cytoplasm were assessed. The responses were compared among treatments using ANOVA, with the comparison of means using the Tukey test. In cases of non-normality, data were log-transformed. Cleavage, blastocyst development and blastomere rates differ among treatments ($P < 0.05$), where T2 and T3 had fewer structures. Both the DCFH and lipid intensity were similar for both cleaved structures and blastocysts ($P > 0.05$). The inclusion of PES in the medium for culture *in vitro* was not effective in reducing the lipid and ROS content in swine embryos.

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FEMALE REPRODUCTIVE BIOLOGY

Distribution of collagen fibers in the endometrium os mares after insufflation with ozone gas vs. oxygen gas

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Uterine inflammatory processes promote an imbalance between metalloproteinases that can result in uncontrolled deposition of extracellular matrix components, such as collagen. The aim of this study was to evaluate the effect of uterine insufflation with ozone gas (O3) vs oxygen gas (O2) on the levels of collagen fibers in the endometrium of mares with endometritis. Thirty non-pregnant mares underwent uterine insufflation with 44 ug/L of O3 (treated group, n=18) or O2 (control group, n=12) at 48-hour intervals, after uterine lavage with 0.9% NaCl solution, totaling 3 insufflations/mare. Endometrial tissue samples were colected before and after the experimental protocols using forceps suitable for uterine biopsy. The samples were fixed in Bouin's solution, immersed in resin and stained with Picrosirius Red. Evaluations were carried out using polarized light microscopy. Microphotographs of 8 fields from each biopsy were randomly obtained for histomorphometric quantification in % of type I and III collagen fibers using ImageJ software. The normality of data was checked by Shapiro Wilk test and the homogeneity of variance by Levene test; the means were compared using Wilcoxon, and all tests were performed with an alpha of 0.05. In the mares of the control group, there was a reduction in the proportion of total collagen fibers ($P<0.05$) after uterine insufflation with oxygen (17.8 ± 6.97 vs 12.18 ± 9.30), while the mares in the group treated with ozone gas showed no change in the amount of total endometrial collagen fibers (14.36 ± 12.14 vs 10.97 ± 9.74; $P>0.05$). The results showed that ozone, at the concentration tested, did not produce any alterations that could compromise or worsen the initial uterine condition, demonstrating the safety of its use in terms of collagen levels in the endometrial extracellular matrix. On the other hand, oxygen insufflation had an effect on the tissue, possibly due to an effect on local oxidative homeostasis that culminated with the degradation of endometrial collagen fibers.

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FEMALE REPRODUCTIVE BIOLOGY

Forskolin supplementation during *in vitro* maturation does not suppress meiotic resumption in cat oocytes

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Intra-oocyte cyclic adenosine monophosphate (cAMP) concentrations decrease when the oocyte is mechanically removed from the antral and precocious meiotic resumption begins. Forskolin has been widely used as a strategy to suppress meiotic resumption since it can regulate cAMP production by activating adenylate cyclase enzyme, keeping its concentration in species such as mice, rats, swine, cattle, and sheep. Several improvements were already reported within forskolin such as the maintenance extension of gap junctions. The domestic cat is considered a reliable reproductive experimental model for endangered wild felids; and the role of forskolin in cat oocytes is still not yet elucidated. For this reason, this study aimed to monitor the effect of different concentrations of forskolin on oocyte nuclear maturation and gap junction activity (GJA) in domestic cats. COCs were recovered from ovaries obtained in elective surgeries and selected based on cytoplasm homogeneity and cumulus cell layers. A total of seven replicates were conducted (n=325 COCs). COCs were allocated into three groups according to the 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) treatment: Control (0 mM forskolin), FK10 (10 mM forskolin) and FK100 (100 mM forskolin). After 6 h of IVM, most COCs (n=295) were denuded with hyaluronidase, fixed in 4% paraformaldehyde, stained with Hoechst 33342, and evaluated under fluorescence microscopy. The remaining COCs (n=30) were submitted to Gap Junctional activity evaluation using calcein staining and measured for fluorescent intensity by ImageJ software. There was no difference between Control and FK10 regarding the GV stage until 6 h of IVM (10.3% vs 13.3%); however, in a higher concentration (FK100), forskolin reduced ($P<0.05$) the GV rate (2.3%) of cat oocytes. Regarding GJA, there was no difference ($P>0.05$) between Control and FK10 (1±0.5 vs 1.2±0.3), while FK100 showed higher activity ($P<0.05$) than the other groups (2.1±0.3). We concluded that 100 mM Forskolin seems to stimulate meiotic progression in cat oocytes, which is, in fact, the opposite behavior observed in other species. However, it also increased the GJA, highlighting the need for more studies on its different actions in this species.

Keywords: biodiversity; cAMP modulator; feline; IVM.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

CellFate-FIV: A 3D culture system to improve *in vitro* oocyte maturation and embryo production

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Despite the success by biotechnology in *in vitro* embryo production (IVP), current commercial conditions in the animal reproduction industry are still suboptimal. In fact, losses in the *in vitro* development of bovine oocytes to the blastocyst stage may reach up to 60 to 70% (1). The low production scenario may be related to the fact that the IVP system currently operates in two-dimensional (2D) cell culturing platforms. Therefore, the three-dimensional 3D culture system emerges as a promising approach to improve the cumulus-oocyte complex (COC) viability and embryo production, as it can provide an environment closer to that of the oviduct. The aim of this study was to evaluate the *in vitro* maturation of oocytes (IVM) and *in vitro* culture of embryos (IVC) using CellFate-FIV, a 3D culture system composed of biocompatible polymer (CellFate®, Biocelltis S.A., Brazil). For this purpose, bovine COCs aspirated from female bovine ovaries were matured *in vitro* using the CellFate-FIV system; part of the matured COCs underwent cellular and molecular evaluations of oocyte competence acquisition and microscopic analysis. Subsequently, the remaining COCs underwent to fertilization and *in vitro* embryo culture in CellFate® (3D-IVC), and the rates of embryo development production were evaluated. CellFate-FIV allowed COCs to mature in the third dimension, and although we didn't find differences in the quantity of matured COC *in vitro*, our 3D system reduces the flattening and preserves COCs structural and functional integrity. After FIV, the cleavage did not differ statistically from 2D to 3D (88.34±4.71 and 81.59±15.56, respectively, p=0.62, unpaired T-test), but we observed differences in cellular behaviour, with expected improvements in embryo differentiation, proliferation, and quality compared to the 2D system. Finally, we anticipate that this may become an useful commercial model for evaluating new products and bioprocesses that can maximize the fertility of mammalian species in assisted reproduction programs.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Protective effect of mangiferin on porcine ovarian tissue against doxorubicin toxicity: an antioxidant approach

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Doxorubicin (DOX) is a chemotherapeutic agent used in cancer treatment. However, its nonspecific action leads to the death of normal cells, including reproductive cells, through oxidative stress [1,2]. Studies with natural antioxidants, such as mangiferin (Mangifera indica L., [3]), have been recommended to overcome these secondary effects of DOX. This study aimed to evaluate the potential protective effect of mangiferin (MAN) during *in vitro* culture of porcine ovarian tissue exposed to DOX. The ovarian cortex of each pair (n = 5) was divided into 18 fragments, with 2 fragments allocated for treatment (uncultured control) and fixed in Carnoy for subsequent histological processing. The remaining fragments were cultured in 24-well plates (2 fragments/well) for 48 h (38.5°C and 7.5% CO2). Fragments were distributed/cultured in 9 treatments: isolated α-MEM+ (cultured control), α-MEM+ supplemented with DOX at 0.3 µg/mL, isolated MAN (10, 50, or 100 µM), or combined with DOX at the same concentrations (MAN10+DOX, MAN50+DOX, and MAN100+DOX). After culture, preantral follicles were morphologically evaluated. Additionally, spent media were collected for analysis of antioxidant capacity (ABTS and DPPH free radicals), with significance level set at $P < 0.05$. In the morphological analysis, the percentage of normal preantral follicles (primordial + growing) was lower ($P < 0.05$) in all cultured treatments compared to the uncultured control. Compared to the cultured control (α-MEM+), the percentage of normal preantral follicles was lower ($P < 0.05$) only in the DOX and MAN 100+DOX treatments. When compared to the DOX treatment, all MAN isolated or combined with DOX treatments had a higher ($P < 0.05$) percentage of normal preantral follicles. Regarding the antioxidant capacity in the collected media, all MAN isolated or combined with DOX treatments had higher ($P < 0.05$) levels of ABTS compared to the cultured control or DOX. For DPPH, the MAN 100 and MAN 10+DOX treatments showed higher ($P < 0.05$) levels compared to the cultured control. Compared to DOX, only MAN 100 had higher ($P < 0.05$) levels of DPPH. Among the MAN isolated or combined with DOX treatments, MAN 10 had lower ($P < 0.05$) levels of DPPH compared to MAN 100. The results reveal that DOX impairs the morphology of porcine preantral follicles, while MAN, whether isolated or combined with DOX, protects the follicles by increasing the percentage of normal follicles. Although MAN does not directly reduce free radicals, its protective role may be linked to its indirect antioxidant capacity, through cellular signaling pathways and structural protection. The increase in stable ABTS and DPPH free radicals suggests greater resistance of ovarian tissue to oxidative stress induced by DOX. *In vivo* studies have shown that MAN reduces lipid peroxidation and increases antioxidant enzymes (SOD and GSH) in hepatic cells of DOX-treated mice, indicating a protective effect [4]. *In vitro* studies have also demonstrated that MAN enhances the efficacy of DOX, reducing the viability of cancer cells [5]. In conclusion, MAN, especially at lower concentrations combined with DOX, exerts a significantly protective effect on porcine ovarian tissue, highlighting its potential to mitigate the adverse effects of chemotherapy on female reproductive organs.

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X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

DIVERGENT MODULATION OF ANTIOXIDANT ENZYMES IN THE OVARIAN TISSUE OF WISTAR RATS AFTER NANDROLONE DECANOATE ADMINISTRATION

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Nandrolone Decanoate (ND) is an anabolic androgenic steroid (AAS) widely used to enhance body aesthetic. The abusive use of this ergogenic agent has been causing numerous reproductive irregularities (1). Recently, our team have demonstrated that ND has been shown to trigger oxidative stress due to the excess in reactive oxygen species (ROS) production, leading to the imbalance of the antioxidant defense system, in testicular tissue (2). However, few studies describe the ND impacts on antioxidant enzymes levels in ovarian tissue. Thus, the aim of this study was to evaluate the effect of ND on the antioxidant enzymes activities, superoxide dismutase (SOD) and catalase (CAT), to predict a possible impact on the redox system in the ovarian tissue of Wistar rats. After approval by the Ethics Committee on Animal Use (CEUA) of the Universidade Estadual do Ceará (UECE), 14 Wistar rats, 14 weeks old, with an average weight of 160g, were used and kept in a 12-hour light/dark cycle, with water and food ad libitum. The animals were divided into Control (Ctrl) and Nandrolone Decanoate (ND) experimental groups. For 7 weeks, rats in the ND group received by intramuscular injection (IM) of 10 mg/kg/week of the AAS, while rats in the Ctrl group received 200 µL of peanut oil by IM. For the evaluation of SOD and CAT enzymes levels, the tissue was initially homogenized and then the total proteins concentration was measured by the Bradford method. For the quantification of SOD activity, the adrenaline auto-oxidation inhibition method was employed, and for CAT, the hydrogen peroxide quantification method, both by spectrophotometry. Data were analyzed using unpaired t-test and results were considered significant when $P<0.05$. As results, it was observed that SOD and CAT enzyme activity were significant increase and decrease ($P<0.05$) in the ND group compared to the Ctrl group, respectively. Supraphysiological doses and prolonged treatments with ND have been related to increased ROS and inhibition of antioxidant enzyme activity in the testicular tissue of rats (3). Such inhibition was reported in the present study by the reduction in CAT activity. However, the increase in SOD may be related to the alternation of expression of the three enzyme isoforms at different stages of folliculogenesis and the ovarian cycle, which requires higher activity to balance the excess of ROS (4). Thus, it is concluded that the administration of ND in rats causes a singular alteration in the activity of SOD and CAT enzymes in ovarian tissue; however, there is a need to further investigate the mechanisms controlling the redox balance in the ovary.

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X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Assessment of antioxidant potential of chlorogenic acid and its effects on follicular morphology, stromal cell density, and thiol levels of bovine ovairan tissue cultured *in vitro*

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The *in vitro* ovarian follicles microenvironment can interfere in the tissue cell composing, potentially causing damage to the follicles. Some alterations may be li nked to the excessive generation of oxidative species that disrupt follicular and stromal homeostasis (1). Chlorogenic acid, found in various plants, is a molecule with high antioxidant activity (2). Because of its chemical structure, it has the potential to promote normal follicular morphology by combating free radicals (3). This study aimed to investigate the radical scavenging activity of chlorogenic acid and its effects on bovine follicular morphology, stromal cell density, and thiol levels in bovine ovarian tissues cultured *in vitro*. The free radical scavenging potential of chlorogenic acid was assessed through DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assays. Fragments of bovine ovarian tissue were cultured *in vitro* in α-MEM+ alone or supplemented with 25, 50, 100 and 200 µmol of chlorogenic acid for 6 days at 38.5 °C and 5% CO2 in air. The uncultured control samples and the fragments of bovine ovarian tissue that were cultivated were fixed in paraformaldehyde (4%). Morphology and stromal density were analyzed by classical histology. For the follicular morphology classification, the follicles were classified as primordial (oocytes surrounded by a flattened layer of granulosa cells), primary (oocytes surrounded by a layer of cubic granulosa cells), and secondary (oocytes surrounded by two or more complete layers of granulosa cells). At the end of the cultivation period, thiol levels were investigated. GraphPad Prism software (9.0) was used to statistically analyze the data. The percentage of normal follicles in each treatment was compared using the chi-square test. Radical scavenging activity and levels, stromal cell density data and thiol levels were analyzed using analysis of variance (ANOVA) and Tukey's test. The association between stromal cell density and the percentage of normal preantral follicles was assessed by linear regression analysis ($P < 0.05$). The results showed that chlorogenic acid has radical scavenging activity, as detected in both the DPPH and ABTS assays, with values of 5.7 ± 0.02 and 5.43 ± 0.01, respectively. The presence of 100 µmol of chlorogenic acid in the culture medium promotes an increase in morphologically normal follicles and stromal cell density in cultured ovarian tissues when compared to those cultured in the control medium. In addition, a positive correlation was observed between the morphology of normal follicles and the increase of stromal cell density. Furthermore, chlorogenic acid increased thiol activity, representing a less oxidative environment. In conclusion, chlorogenic acid exhibits radical scavenging activity, and 100 µmol/L chlorogenic acid increases morphologically normal follicles, stromal cell density, and thiol levels in cultured bovine ovarian tissues.

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FEMALE REPRODUCTIVE BIOLOGY

Body's energy reserve affects extracellular vesicles and uterine cells from the uterotubal junction of Nelore cows

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Nutritional plan or body conditions changes can lead to alterations in the concentration of hormones and metabolites which are crucial for bovine females reproduction. Animals with high body energy reserve (BER) exhibit reduced embryonic recovery on the 4th day of embryonic development compared to those with moderate energy reserve (1). During the pre-implantation period, the embryo relies solely on uterine secretions produced by maternal endometrial cells. In addition to the conventional mode of cellular communication via direct cell-to-cell contact, extracellular vesicles (EVs) have been identified in uterine fluid and recognized as a novel mediator of maternal-embryonic communication (2). These EVs utilize extracellular fluids to disseminate and transfer their contents to target cells, thereby modulating function through the delivery of bioactive materials such as mRNAs, miRNAs, proteins, among others (3). However, little is known about the influence of increased BER on the endometrial receptivity of the uterotubal junction (UTJ), the place where the embryo establishes its first contact with the maternal endometrium. The aim of this study was to evaluate the effects of increased BER in miRNA and mRNA profile of EVs and endometrial cells from UTJ of cattle, respectively. For this, Nelore cows from the same herd were submitted to different nutritional plans during 67 days of feedlot, in order to maintain (MBER group; n=3) or increase (HBER group; n=3) their BER. At the end of the feedlot period, animals were submitted to estrous synchronization, artificial insemination and were slaughtered approximately 120 hours after ovulation induction. The reproductive tracts were collected, the UTJ ipsilateral to the corpus luteum were dissected and flushed with 2 mL of 1xPBS free of Ca2+ and Mg2+. After this, the UTJ cells were collected by obtaining endometrial fragments. For this study we used only the UTJ samples from animals in which an 8-cell embryo was found in the reproductive tract. The EVs were isolated from de UTJ fluid by ultracentrifugation and analyzed based on particle size and concentration by nanoparticle tracking analysis (NTA). No differences were identified in mean mode size (MBER: 139.2 ± 2.76 nm; HBER: 145 ± 5.66 nm) and particle concentration (MBER: 2.76 x 109 ± 8.17 particles/mL x 107; HBER: 3 x 109 ± 1.52 x 108 particles/mL) between the groups. The total RNA of EVs was extracted using QIAzol and the miRNAs was analyzed for the relative expression levels of 382 miRNAs. The results demonstrated 42 miRNAs commonly expressed in both groups, of which 9 were differentially expressed (DE) and all downregulated in the HBER group. The data obtained were normalized and compared using Student's t-test and the significance level used was 5%. The total RNA content of endometrial cells was extracted using the commercial kit miRNeasy Mini Kit (QIAGEN) and sequencing libraries were prepared using the TruSeq Stranded mRNA Kit (Illumina). According to the results obtained, 435 differently expressed genes (DEG) were identified between the groups, of which 186 are upregulated in the HBER group and 249 are downregulated in the HBER group. Functional enrichment analysis of up-regulated genes revealed the regulation of biological pathways associated with cellular metabolism of glucose, fatty acids and drugs. Functional enrichment analysis of downregulated genes revealed the regulation of biological pathways associated with motor proteins that make up cellular structure. From the results, it is possible to conclude that the HBER is capable of influence the dynamics of the uterine environment, modulating the miRNA profile of EVs in the uterine fluid and the transcript profile of UTJ cells. Thus, these results suggest that cattle with HBER might not have a favorable environmental for early embryo development at UTJ.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Occurrence of virulence genes in *Escherichia coli* strains isolated from cases of acute endometritis in mares

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The role of Escherichia Coli in the pathogenesis of equine endometritis is largely unknown. It is suggested that E.coli induces a less exudative inflammatory response and causes greater damage to endometrial tissue than S. nonepidemic. E. coli infections are reported to be the most problematic to resolve. Determining the virulence factors of E. coli can be a key point in characterizing strains that induce a greater inflammatory response and that, according to their antigenic composition, have a self-defense behavior, making them difficult to isolate and even eliminate from the uterine environment by cellular defense mechanisms. The aim of this study was to verify the association E. coli virulence genes and endometritis in the mare. To this end, six strains of E.coli isolated from endometritis in mares were isolated. The results obtained were through bacterial isolation, gynecological examination of mares, and later extraction of Escherichia Coli DNA. Experimental infection of mares performed and clinical evaluation and evolution was determinantes by identification of endometritis signs together with ultrasound, cytological and bacteriological examination until clinical signs disappeared. The DNA samples were subjected to search of the virulence genes Stx1, Stx2, eaeA, ehxA, hlyA, iuc, ibeA, FimH, KpsMII through Polymerase Chain Reaction (PCR) and *in vitro* biofilm detection.The percentage of prevalence of the genes were: Stx1 (28.57%); Stx2 (57.14%); eaeA (14.28%); ehxA (14.28%); hlyA (28.57%); iuc (28.57%); ibeA (14.28%); fimH (85.71%); KpsMII (14.28%). Strain I - iuc; strain II - Stx2, hlyA and fimH; strain III - Stx2 and fimH; strain IV - Stx1, eaeA, iuc, and fimH; strain V - Stx2, hlyA, ibeA, fimH, KpsMII; strain VI presented only the fimH gene; and strain VII identified the Stx1, Stx2, ehxA, fimH genes. In terms of biofilm formation *in vitro*, strain VI had slight biofilm production, and strain V strong biofilm production. These isolated strains were experimentally inoculated into 6 different clinically normal mares with negative cytological and bacteriological examinations. One day after infection, a clinical examination of the genital tract was carried out, including vaginoscopy, ultrasound, endometrial cytology and bacteriology. The endometritis caused by E.coli caused positive cytology and the mares developed clinical vaginal signs of endometritis and uterine fluid. O fimH virulence factor gene was the most prevalent and significant among E.coli in equine intrauterine infection. No However, only the strain with the presence of the KpsMII gene showed strong biofilm production. In interpreting the phylogenetic group of the E.coli samples studied, they belong to groups A and B1, and are therefore commensal strains. Os data presented in this study indicate that E.coli isolates recovered from the uterus of mares show a wide genetic diversity.

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FEMALE REPRODUCTIVE BIOLOGY

Mosaicism in a Mangalarga Marchador mare: case report

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Cytogenetics is the science that studies the structure, appearance, and performance of chromosomes, which are structures formed by DNA found in the nucleus of somatic cells. Cytogenetic analysis determines the genetic sex, allowing the identification of chromosomal changes, which can be either numerical and/or structural. The mosaic karyotype 63,XO/64,XX, or monosomy of chromosome X, is the third most common anomaly and may have pre- or post-zygotic origin. Found only in sex chromosomes, the loss of an entire chromosome – condition XO – results in an unbalanced genome. Mosaicism is associated with estrous behavior failures, reproductive abnormalities, and infertility and is a genetic modification of cells that belong to the same individual. Animals that present this anomaly are commonly described as small for their breed and age, with small and smooth ovaries devoid of follicles and germ cells. Mares generally present normal morphological external genitalia but have a small and flaccid uterus on rectal palpation due to a deficiency in the circulation of ovarian steroids. A blood sample was collected and sent to the genetic laboratory at Unesp Botucatu, Brazil, for cytogenetic analysis. Disposable syringes and needles were used. Sodium heparin was used to moisten the entire inner wall of the syringe, and 0.2 mL of the anticoagulant was kept homogenizing with 4 mL of venous blood. Blood collection was carried out in the most aseptic way possible to avoid contamination by bacteria, thus preventing cell growth. A five-year-old Mangalarga Marchador mare was examined by an Equine Reproduction Veterinary Specialist with a clinical history of never cycling or being observed in heat and never having any follicular growth when evaluated by ultrasonography. The mare was physically smaller than expected for the age and breed, and the ultrasound evaluation showed the presence of small ovaries and an underdeveloped reproductive tract (internal genital) with a low cervical tonus (flaccid cervix). There was a presence of a few numbers of small follicles at the ovaries, among 5 and 10 mm in diameter. In lymphocyte culture, 35 metaphase cells were analyzed using conventional GIEMSA staining. The presence of only one X chromosome was observed in 29% of the cells, thus proving the karyotype abnormality and the reason for the animal's infertility. The animal thus had to be removed from the breeding program, and no further expectations to obtain products from this animal were created. Acknowledgments: JMG Souza-Fabjan is a FAPERJ and CNPq fellow.

Keywords: cytogenetics; equine; infertility.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

In vitro maturation of bovine cumulus-oocyte complexes in presence of follicular fluid extracellular vesicles: effects on oocyte maturation and developmental competence, and their contents associated with epigenetic regulation, preliminary results

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In vivo, bovine oocytes develop and mature within the follicular fluid (FF), which contains a variety of components including extracellular vesicles (EVs). Such vesicles carry different molecules, including mRNAs, which can regulate cellular function of target cells. However, during *in vitro* maturation (IVM), oocytes are matured in the absence of follicular EVs (fEVs). Therefore, the aim of the present study was to investigate effects of fEVs during IVM on oocyte maturation and developmental competence and to determine their contents regarding epigenetic regulation transcripts, which could potentially impact function in cumulus-oocyte complexes (COCs). Bovine FF and COCs were obtained from slaughterhouse ovaries by aspiration of 3-8mm follicles from separate collections. fEVs were isolated from 1 ml FF by size exclusion chromatography (SEC, qEV1 columns 35 nm Gen 2, Izon) followed by ultracentrifugation (100,000 x g for 70 min at 4°C, Beckman Coulter), and used for mRNA analysis content by RT-PCR or for medium supplementation during IVM. Bovine COCs were subjected to IVM in TCM199 containing 0.4 mM glutamine, 0.2 mM pyruvate, 50 mg/mL gentamicin, EGF (20 ng/ml) and supplemented with 10% fetal calf serum (FCS, control) or 10% FCS depleted of its own EVs and added with fEVs (fEVs group), for 24h, at 38.5oC and 5% CO2 in air. At the end of IVM, cumulus cells were removed from part of the COCs, and denuded oocytes evaluated for maturation rates by first polar body extrusion (1st PBE, 3 replicates), while cumulus cells (CC) were assessed for epigenetic regulation transcripts by RT-PCR (3 replicates). The other part of COCs was submitted to *in vitro* fertilization and culture, and blastocyst rates on day 7 (D7) were recorded (3 replicates). The Wilcoxon two group test was used to analyze the rates of maturation, blastocyst and ΔCt values. Maturation rates were not affected by treatments (P>0.05) and 1st PBE rate was 77 (n=204) and 76% (n=203), for control and fEVs, respectively. D7 blastocyst rates were also unaffected (37 and 36%, respectively, P>0.05). fEVs were shown to contain transcripts for DNMT1 (Ct = 30.53), DNMT3A (Ct = 31.29), MAT2A (Ct = 30.11), and SHMT2 (Ct = 29.05), which were also expressed in CC, but the average expression levels did not vary between studied groups [P>0.05; control CC DNMT1 (ΔCt = 3.42), DNMT3A (ΔCt = 4.59), MAT2A (ΔCt = 1.08), and SHMT2 (ΔCt = 0.67) and fEV treatment DNMT1 (ΔCt = 3.30), DNMT3A (ΔCt = 5.38), MAT2A (ΔCt = 2.24), and SHMT2 (ΔCt = 1.72). Considering present results, it may be concluded that fEVs do not improve maturation and embryo development; although CC transcripts levels for epigenetic regulators were also unaffected, as the fEVs carry such transcripts, effects on epigenetic regulation in oocytes cannot be ruled out. This study shows preliminary results, and expression in treated oocytes is ongoing, as well as the analysis of other epigenetic regulators, which may bring further knowledge to the area. Financial support: FAPESP (SPEC Grant # 2021/09886-8; AR Grant #2021/06760-3); FS - DS Scholarship (Capes 88887.694635/2022-00); AB - PD Scholarship (FAPESP 2023/01524-5); JRQO - ScI Scholarship (FAPESP 2023/12424-1); LCZJ DS Scholarship (Capes 88887.836321/2023-00); LCM - ScI Scholarship (PUB-USP 2023/83-1).

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Gallic acid promotes the development of preantral follicles and reduces apoptosis after *in vitro* culture of sheep ovarian tissue

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Oxidative stress can negatively impact follicular quality during *in vitro* culture [1], necessitating the addition of antioxidants to the culture medium, such as gallic acid (GA), a phenolic compound naturally present in various plants and fruits residues commonly found in the Semiarid region, such as grapes and bananas [2]. However, there are no reports on the effects of GA on the *in vitro* culture of ovarian tissue in any species. Therefore, the aim of this study was to evaluate the effect of GA as the sole antioxidant added to the standard culture medium of sheep ovarian tissue, replacing transferrin, selenium, and ascorbic acid. Fragments of the ovine ovarian cortex were immediately fixed for histological analysis (fresh control) or cultured for 7 days in α-MEM supplemented with 10 ng/ml insulin, 2.5 mg/ml transferrin, 4 ng/ml selenium, 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/mL bovine serum albumin and 50 µg/ml ascorbic acid (control; α-MEM+) or in α-MEM+ supplemented with 25, 50 or 100 µM GA (replacing the antioxidants transferrin, selenium, and ascorbic acid). At end of the culture period, the fragments were fixed and histologically analyzed to determine the percentage of morphologically normal follicles (survival), activation of primordial follicles, and follicular growth. Subsequently, immunostaining for activated caspase-3 (apoptosis) and proliferating cell nuclear antigen (PCNA) was performed in the treatments with the most desirable outcomes (α-MEM+ and 50 µM GA). Furthermore, to evaluate the antioxidant potential of these media, the ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation decolorization test was carried out. After culture, treatment containing 50 µM GA increased ($P<0.05$) survival (76.67%) and reduced ($P<0.05$) apoptosis (46.67%) compared to α-MEM+ (64.40% normal follicles and 70% apoptosis). In a previous study, supplementation of the *in vitro* culture medium with GA maintained the survival of isolated ovine secondary follicles [3]. In addition, the capacity to eliminate the ABTS radical was greater in the medium containing 50 µM GA (9.32 mg ascorbic acid/L) compared to α-MEM+ (8.59 mg ascorbic acid/L). Therefore, we suggest that the antioxidant activity of GA (50 µM) increased follicular survival *in vitro*, reducing apoptosis. No differences ($P>0.05$) were observed in the activation of primordial follicles among the different treatments. However, 50 µM GA increased ($P<0.05$) follicular growth (26.35 µm) and cell proliferation (64.16%) compared to α-MEM+ (23.52 µm; 37.19% PCNA positive cells). This proliferative effect of GA on granulosa cells has also been described in mouse ovaries [4]. In conclusion, GA at 50 µM is more effective than a combination of three antioxidants (transferrin, selenium, and ascorbic acid) during the *in vitro* culture of sheep ovarian tissue, supporting follicular survival and reducing apoptosis, while enhancing follicle growth and cell proliferation. Therefore, using GA to replace other antioxidants in *in vitro* follicular culture could reduce the costs of this biotechnique. Furthermore, fruit residues containing GA can be incorporated into the diet of sheep with the aim of enhancing follicular development.

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FEMALE REPRODUCTIVE BIOLOGY

Effect of resveratrol on toxicity induced by doxorubicin in the *in vitro* culture of goat secondary follicles

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Doxorubicin is a chemotherapy drug commonly used in the treatment of solid tumors, leukemia, and lymphomas. However, it can cause a toxic effects on the ovary [1]. Resveratrol is a polyphenol with antioxidant properties [2] that can alleviate the toxicity of doxorubicin. Therefore, the aim of this study was to evaluate the effect of resveratrol against doxorubicin-induced toxicity during *in vitro* culture of goat secondary follicles. Follicles were isolated and cultured for 6 days in control medium (α-MEM+) or in α-MEM+ supplemented with doxorubicin, different concentrations of resveratrol (5 or 10 µM) or a combination of both resveratrol concentrations and doxorubicin. The endpoints analyzed included survival, antrum formation, diameter and growth rate, assessed on days 0, 2, 4, and 6 of culture. After six days of culture, the levels of glutathione (GSH) and mitochondrial activity were analyzed. The groups cultured with 5 and 10 µM of resveratrol alone, and with 10 µM of resveratrol associated with doxorubicin, maintained (P>0.05) the percentage of normal follicles (88.24%, 91.9%, 96.88% and 92.88%, respectively) compared to α-MEM+. The follicles cultured with doxorubicin alone showed reduced (P>0.05) follicular growth, mitochondrial activity, and GSH levels. This result suggests that doxorubicin may act by increasing oxidative stress, reducing mitochondrial activity, thereby decreasing follicular development and reducing follicular growth [4]. However, the group treated with 5 µM of resveratrol associated with doxorubicin showed a decrease (P>0.05; 82.86% normal follicles) in follicular survival. It is likely that this concentration of resveratrol is insufficient to protect the follicle from the action of doxorubicin, as observed in the study by Nishigaki et al., 2022, where administration of 10 mg/kg of resveratrol against cisplatin promoted an increase in markers of oxidative stress in the uterus of mice [5]. Furthermore, the groups treated with resveratrol in association with doxorubicin showed a reduction (P>0.05) in the percentage of antrum formation compared to α-MEM+ and doxorubicin. However, the association of 10 µM of resveratrol with doxorubicin increased (P>0.05) the overall growth rate compared to the group treated with 5 µM associated with doxorubicin. Only the group treated with 10 µM of resveratrol maintained mitochondrial activity and GSH levels similar to the control medium. It is possible that resveratrol exerts a protective effect by increasing the expression of SIRT-1, which can modulate ovarian functions through the activation of the gonadotropin receptor [6]. In conclusion, supplementation of the culture medium with 0.2 µg/mL doxorubicin impaired follicular growth and reduced mitochondrial activity and GSH concentrations. Resveratrol at 10 µM maintained follicular survival, mitochondrial activity and GSH levels, attenuating the toxic effects of doxorubicin.

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X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Punicalagin contributes to the activation and development of primordial follicles and increased antioxidant activity during *in vitro* culture of bovine ovarian tissue

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Culturing preantral follicles within ovarian tissue *in vitro* is a valuable tool for investigating early follicular development. Despite various ovarian tissue culture systems having been tested, oxidative stress remains a significant concern. Elevated levels of reactive oxygen species (ROS) can impair endogenous antioxidant defenses, including the activities of key enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). In response to this challenge, natural antioxidant substances like Punicalagin have emerged as promising candidates for supplementation in culture media. This study aimed to assess the impact of different concentrations of Punicalagin on follicle activation and development, as well as the activity of antioxidant enzymes SOD, CAT, GPX, and thiol levels. Bovine ovaries were obtained from a local slaughterhouse, sanitized, and fragmented in the laboratory (3x3x1) before being cultured in 24-well plates for 6 days in either α-MEM+ control medium or medium supplemented with varying concentrations of Punicalagin (1, 10, or 100 µM). At the conclusion of the experimental period, a portion of the ovarian fragments underwent histological processing and staining with hematoxylin and eosin. For biochemical analysis, the Bradford method was employed on 100 mg/ml samples of ovarian tissue per treatment. These samples were macerated in phosphate buffer, centrifuged, and subsequently subjected to spectrophotometric analysis to measure the activity of SOD, CAT, GPX enzymes, and thiol levels. The enzymatic activity of SOD, CAT, GPX and thiol levels were evaluated using the ANOVA and Kruskal-Wallis tests. Results revealed a significant reduction in the percentage of primordial follicles in ovarian tissues cultured across all treatments. However, the presence of Punicalagin (at concentrations of 1, 10, or 100 µM) led to an increase in the percentage of developing follicles compared to tissues cultured in the control medium ($P < 0.05$). Furthermore, ovarian tissues cultured with 10 µM Punicalagin exhibited significantly higher levels of thiol, alongside increased activity of SOD, CAT, and GPX enzymes, compared to those cultured in the control medium ($P < 0.05$). Then, we can conclude that Punicalagin added to the *in vitro* culture solution improved the pro-oxidant environment by increasing the levels of thiols along with the activity of the SOD, CAT and GPX enzymes, making it a viable compound with antioxidant action.

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FEMALE REPRODUCTIVE BIOLOGY

QUALITATIVE METABOLOMIC PROFILE OF FOLLICULAR FLUID FROM FOLLICLES IN SHEEP AND GOATS

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Follicular fluid (FF) is formed from the transudation of theca and granulosa cells into the growing follicular antrum. Its main function is to provide an ideal intrafollicular microenvironment to modulate oocyte maturation. In this sense, the objective of this study was to determine the metabolomic profile of FF from sheep and goats collected from follicles of different sizes. Ovaries from 18 adult sheep and 34 adult goats were collected from a local slaughterhouse and transported at 37°C to the laboratory in 0.9% saline with antibiotics. The ovaries were washed three times in transport solution at 37.5°C and kept in a water bath. Subsequently, with the aid of a caliper, the size of the follicle was determined. FF was collected from small follicles (<3 mm, n=27 and 51) and large follicles (≥3 mm, n=19 and 36) of sheep and goats, respectively, using a 20 G needle attached to a 5 gauge syringe mL. Samples were pooled and centrifuged at 500 G at 4°C for 15 min. Then, the supernatant was recovered and stored in tubes at -80°C until analysis. Subsequently, FF samples (150 µL) from each group were analyzed in triplicates by high-resolution nuclear magnetic resonance spectroscopy (1H NMR). The data were qualitatively evaluated using the R 3.6.2 software. Eighteen metabolites were found in both goat and sheep FF, including amino acids, carbohydrates and intermediate metabolites. Among the metabolites, small sheep follicles showed the highest spectral signal for alanine, choline, choline phosphate (CP), glycerophosphocholine (GPC), myo-inositol and glycerol. While in large follicles there was a greater spectral signal for CP and GPC. For goats, FF from large follicles showed higher spectral signal for alanine, acetic acid, CP, GPC, creatine, choline, betaine, myo-inositol, glycerol. In small follicles, FF showed greater spectral signal for N-acetylated glycoprotein (GNA), acetic acid, glycine, creatine and lactic acid. All of these metabolites found with greater spectral signal are related to the ability to buffer high-energy phosphate, thus contributing to intracellular energy homeostasis (1). Their absence is an indication of low oocyte competence, regardless of follicular size (2). Based on the data, it is concluded that there is physiological variation regarding the metabolism that occurs in follicles in line with the species and size of the follicle. Such data can assist in the development, species-specific, of means for *in vitro* production of embryos.

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FEMALE REPRODUCTIVE BIOLOGY

In vitro effect of Withanolide D on the development of secondary follicles in mice

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Recent advances in cancer diagnosis and therapies have led to more survivors, but concerns persist over the side effects of anticancer treatments, particularly their impact on ovarian health (1). New studies aim to develop more effective drugs while reducing toxicity in normal tissues, like the ovaries. Natural substances, such as Withanolide D (WD), extracted from Acnistus arborescens, are being investigated for their potential to target multiple aspects of cancer cell growth and differentiation (2). In this regard, we investigated whether WD could affect the development of secondary follicles cultured *in vitro*. Secondary follicles were mechanically isolated and individually cultured in half area 96-well plates at 37°C, 100% humidity, 5% carbon dioxide, and normal oxygen, for 12 days. The follicle culture medium consisted of α-minimal essential medium (Invitrogen) supplemented with 5% heat-inactivated fetal bovine serum, 5 mg/ml of insulin, 5 mg/ml of transferrin, 5 ng/ml of selenium (ITS; Sigma, Bornem, Belgium), and 10 IU/l of recombinant follicle-stimulating hormone (r-FSH; Gonal-Fw, Serono, Benelux). The follicles were distributed into the following treatments: Control, DMSO, TAXOL 1 µM, WD 100 nM, and WD 1 µM. Our results show that WD (100nM and 1µM) did not affect the survival of secondary follicles after 12 days of cultivation. According to a previous study by our team, we observed a reduction in the percentage of pre-antral follicles in goats after exposure to WD at concentrations of 1.5, 3.0, and 6µM for 6 days of cultivation (3). However, in our study, secondary follicles were cultured at lower concentrations (100nM and 1µM) and in a different species. Nonetheless, lower percentages of antrum formation and extrusion of the first polar body were observed when we cultivated the secondary follicles individually at lower concentrations of 100nM and 1µM. This study investigated for the first time the toxic effects of whitanolide d on *in vitro* cultured mouse secondary follicles. We conclude that WD could be a promising candidate for chemotherapy, as it does not affect the development of secondary follicles.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Influence of leptin on the *in vitro* maturation of oocytes from sheep preantral follicles

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In vitro embryo production depends on oocytes from antral follicles, which are present in low number in the ovary. In this manner, the utilization of preantral follicles can be an alternative for supplying a large number of competent oocytes fertilizable [1]. However, the percentage of mature oocytes from *in vitro* grown preantral follicles remains low [2], and further improvement of oocyte maturation conditions is required. For example, adding hormones such as leptin to the *in vitro* maturation (IVM) medium could enhance the process, as observed during IVM of oocytes from goat and sheep antral follicles, which increased oocyte maturation [3,4]. However, there are no reports on the effects of leptin on the IVM of sheep oocytes from *in vitro* grown preantral follicles. Thus, the aim of this study was to examine the effect of leptin on the IVM of sheep oocytes from secondary follicles cultured *in vitro*. These follicles were cultured for 12 days in alfa-modified Minimum Essential Medium (α-MEM), added with 50 ng/mL ascorbic acid, 2 mM glutamine, 2 mM hypoxanthine, 10 ng/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL selenium, 3 mg/mL bovine serum albumin (BSA) and 750 ng/mL recombinant follicle-stimulating hormone. Follicular survival, growth, and antrum formation were assessed on days 6 and 12. After culture, the cumulus-oocyte complexes were recovered, and oocytes ≥ 110 µm were matured for 36 hours in 100 µL drop containing tissue culture medium 199 supplemented with 10% fetal bovine serum, 1 µg/mL follicle stimulating hormone, and 1 µg/mL luteinizing hormone (control medium), or in control medium with 10 or 25 ng/mL leptin. Next, nuclear chromatin configuration, mitochondrial activity, and DNA fragmentation, were analyzed. Addition of 25 ng/mL leptin significantly increased the oocytes meiosis resumption (81.3%) compared to other treatments (α-MEM - 42.4% and 10 ng/mL leptin - 38.7%). Some studies have shown that leptin can stimulate meiosis resumption by activating the mitogen-activated protein kinase pathway (MAPK), thereby increasing the percentage of mature oocytes. When activated, the MAPK stimulates cell proliferation, differentiation, and survival, in addition to stimulate cell cycle and cell division processes such as meiosis, thus directly promoting meiotic resumption [3,5]. In addition, our results showed 25 ng/mL leptin significantly increased mitochondrial activity compared to 10 ng/mL and to control medium. Furthermore, leptin supplementation at 25 ng/mL significantly reduced oocyte DNA fragmentation (14.81%) compared to the control (47.06%). Leptin can increase the expression of sirtuin 1, an enzyme that is related to increased mitochondrial activity and protection of this organelle against oxidative stress and mitochondrial dysfunction. This effect may have led to less DNA fragmentation, as mitochondrial ROS production can induce DNA fragmentation [6,7]. In conclusion, supplementing the IVM medium of sheep oocytes from *in vitro* grown secondary follicles with 25 ng/mL of leptin increases the resumption of meiosis, mitochondrial activity, and reduces oocyte DNA fragmentation. This may lead to an increase in the number of matured oocytes, potentially providing a larger quantity of embryos and improving *in vitro* reproduction.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Visfatin enhances survival and promotes the activation of primordial follicles after *in vitro* culture of sheep ovarian tissue

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Visfatin is an adipokine involved in cellular metabolism responses, as well as inflammation and oxidative stress control [1]. In association with insulin-like growth factor-1 (IGF-1), visfatin increased the secretion of estradiol in human and bovine granulosa cells cultured *in vitro* [2,3] and the release of progesterone in buffalo luteal cells [4]. However, there are no studies on the effects of visfatin on the *in vitro* culture of ovarian tissue in any species. Therefore, the aims of this study were to analyze the effects of visfatin on survival, primordial follicle activation, granulosa cell proliferation, and expression of tumour necrosis factor-α (TNFα – inflammation marker) in preantral follicles after *in vitro* culture of sheep ovarian tissue. Ovarian fragments were fixed for histological analysis (fresh control) or cultured in α-minimum essential medium alone (α-MEM+: control medium) or in α-MEM+ supplemented with different concentrations of visfatin (1 or 10 ng/mL) for 7 days. Thereafter, immunohistochemistry was performed to evaluate granulosa cell proliferation and TNFα expression. The results showed that treatments with visfatin maintained the percentage of morphologically normal follicles similar ($P>0.05$) to the fresh control and significantly higher than α-MEM+. A significant increase in primordial follicle activation was observed after 7 days of culture at both visfatin concentrations (90% of growing follicles) compared to fresh control (10% of growing follicles) and α-MEM+ (68% of growing follicles). In addition, only the treatment containing 10 ng/mL of visfatin significantly increased follicular and oocyte diameters compared to α-MEM+, as well as increasing ($P<0.05$) granulosa cell proliferation compared to fresh control and α-MEM+, and attenuated inflammation (reduced TNFα expression) after *in vitro* culture. Administration of visfatin to aged mice increased the total number of surviving follicles (primordial, primary, secondary, and antral follicles) compared to the group not treated with visfatin [5]. *In vivo* and *in vitro* approaches of ischemia/hypoxia, visfatin effectively reduced the expression of inflammatory factors, such as interleukin-1β (IL-1β) and TNF-α, and further apoptosis of myocardial cells in rat model [6]. Thus, in the current study, it is possible that visfatin maintained follicle survival by reducing inflammation and potentially decreasing oxidative stress and subsequent apoptotic events. Results from previous studies have demonstrated that exogenous visfatin promotes the proliferation of rat cardiac fibroblasts [7]. In addition, visfatin has a stimulatory effect on the secretion of angiogenic factors, such as vascular endothelial growth factor (VEGF) and stromal cell-derived factor 1 (SDF-1α) from ovarian stromal cells and granulosa cells *in vitro*, which are intraovarian factors that may play an important role in follicular growth and development [5]. Therefore, visfatin is likely acting on primordial follicle activation by stimulating the proliferation of granulosa cells and the subsequent secretion of other stimulatory factors, leading to the growth of the oocyte and follicle. In conclusion, 10 ng/mL visfatin maintains survival, reduces inflammation and promotes the activation of primordial follicles by stimulating granulosa cell proliferation after *in vitro* culture of sheep ovarian tissue.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

IN VITRO EFFECTS OF WHITAFERIN A IN HEALTHY MICE OVARIES, AFTER TWO FREQUENCES OF EXPOUSURE

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Withaferin A (WTA) is a naturally occurring compound from the withanolide family, known for its inhibitory effect on the proliferation of various cancer cell lines both *in vitro* and *in vivo*, making it a potent candidate for chemotherapy. However, despite extensive research on its antitumor properties, it's *in vitro* effects on the survival and development of healthy preantral follicles are not yet fully understood. Therefore, the objective of this study was to evaluate the effects of WTA exposure at different concentrations on preantral follicles of mice during *in vitro* culture. Female mice (n=40) were sacrificed by overdose of ketamine/xylazine solution, and the ovaries were collected, dissected, and randomly allocated into 8 groups: uncultured control (CTR); cultured in α-MEM base medium supplemented with 1% Dimethyl Sulfoxide (DMSO); Doxorubicin 0.3 µg/ml (DOXO); WTA 0.6 µM; or WTA 6 µM. Regarding exposure to DOXO and WTA (0.6 and 6 µM), this was done at two different periods: only on day 0 of culture (1X); or on days 0, 2, and 4 (3X) during *in vitro* culture. Thus, the following experimental treatments were generated: CTR; DMSO; DOXO (1X); DOXO (3X); WTA 0.6 µM (1X); WTA 0.6 µM (3X); WTA 6 µM (1X); WTA 6 µM (3X). The ovaries were cultured at 37°C and 5% CO2 in air for 6 days, with complete medium change every 2 days. After the *in vitro* culture period, the ovaries were fixed and processed for morphological analysis. Statistical analysis was performed using ANOVA and post hoc Fisher and t-Student tests, with results considered significant when $p<0.05$. Regarding morphological analysis, when exposed only once, WTA 6 increased ($p<0.05$) the percentage of degenerated follicles compared to WTA 0.6 and DOXO. However, these same drugs when exposed three times showed no differences between them ($P>0.05$). The percentage of primordial follicles exposed only once was reduced ($P < 0.05$) in the DOXO and WTA 6 treatments compared to the DMSO treatment. Moreover, when comparing exposures of 1X and 3X, only the WTA 0.6 treatment reduced ($p<0.05$) the percentage of primordial follicles. Regarding developing follicles, there was an increase ($P<0.05$) in the DOXO (1X) and WTA 0.6 (3X) treatments compared to the control and DMSO. Furthermore, there was a reduction ($P<0.05$) in the percentage of developing follicles in the WTA 6 (3X) treatment compared to WTA 0.6 (3X). Finally, the stromal cell density was ($P<0.05$) reduced in the WTA 0.6 (1X) and WTA 6 (1X) treatments compared to the CTR and DMSO. These results are consistent with those obtained by Kakar et al.,[2], demonstrating that at higher concentrations, WTA was more toxic. Thus, we observe and suggest that the toxic effects of WTA are dose- and exposure frequency-dependent. Therefore, in addition to the toxic effects of withanolide compounds on cancer cell lines, these molecules demonstrated toxicity on the morphology and development of healthy preantral follicles [3]. Thus, we conclude that WTA demonstrated ovarian toxicity similar to DOXO, a widely used anticancer agent. Furthermore, the higher the concentration (WTA 6) and exposure frequency (3X), the greater the cytotoxic effects.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Comparison between four preparation protocols Platelet-Rich Plasma in the equine species

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Platelet-rich plasma (PRP) is a blood derivative obtained through centrifugation, with the objective to separate plasma and red blood cells into heterogeneous phases, aiming to obtain a supraphysiological platelet dose. Its therapeutic effect derives from the immunomodulatory properties promoted by growth factors (GF) with biological activity and cytokines, which stimulate angiogenesis and tissue repair. The PRP can decrease inflammatory response after artificial insemination and increase fertility in mares with chronic degenerative endometritis. Six Quarter Horses, weighing an average of 400kg, aged between 6 and 16 years, were selected, with a total blood platelet count greater than 100,000 platelets/µL. Blood was collected by venipuncture of the external jugular, using 21G vacuum needles and tubes with 3.2% sodium citrate anticoagulant. To obtain the PRP, four distinct protocols (P) were established, based on the comparative principles available in literature. The centrifugation protocols (P1, P2, P3, and P4) were taken, respectively, from Muthu et al., (2022), Seidel et al., (2019), Segabinazzi et al., (2021), and Miranda et al., (2019). P1: 1st Centrifugation 100G/15 min and 2nd Centrifugation 1,600G/20min; P2: 1st Centrifugation 300G/5min and 2nd Centrifugation 700G/15min; P3: 1st Centrifugation 400G/15 min and 2nd Centrifugation 1,000G/10min; P4: 1st Centrifugation 120G/10 min and 2nd Centrifugation 240G/10min. The standardization of PRP occurred after the second centrifugation, with a rest period of 25 minutes between centrifugations, 1/3 of the upper volume of the tube was discarded and pipetted 1 ml above the leukocyte layer and 2-6 mm below. The quantification of the PRP sample was performed in a Neubauer hematimetric chamber, using Brecher method, which is considered the gold standard. For statistical analysis, multiple comparison of the means was performed using the Turkey test. In all protocols, the increase in platelet concentration proportional to the initial count in whole blood was evaluated. Platelet enrichment when compared to whole blood was achieved in protocols P1, P3 and P4. The P2 protocol did not show significant difference ($P>0.05$) in enrichment when compared to whole blood count, demonstrating that in the present study this protocol was not efficient in concentrating platelets. In addition, the results obtained from P1 and P2 did not reach a significant difference between them ($P>0.05$). The present study described procedures that produce PRPs, covering only the platelet concentration that can estimate a proliferative activity. In this study, only protocols 3 and 4 demonstrated efficiency in producing PRP with high platelet enrichment.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Color Doppler ultrasonographic evaluation of ovarian tissue transplantation in goats

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In ovarian tissue transplantation, there is a search for a suitable location that provides better revascularization of the transplanted tissue, favoring an adequate environment and less damage after tissue grafting, seeking an *in vivo* method that can encompass pre-pubertal women and young people with oncological problems, who face chemotherapy treatments, predisposing them to pre-mature ovarian failure and consequent infertility (1; 2). The objective of this work was to test a new implant site Intra-Auricular Subcutaneous (IA), in comparison with Intramuscular (IM) in the cervical portion of the neck in goats. Four fragments of ovarian cortex were allotransplanted at sites IA and IM and recovered 7 (IA-7; IM-7) and 15 (IA-15; IM-15) days after grafting. All imaging examinations were performed by the same individual using a duplex portable color Doppler ultrasound device (M6VET, Mindray, Schenzhen, China) connected to a 5.0 to 10 MHz convex probe. Ultrasound video clips (≤10 sec) were obtained at allograft transplant sites. For all ultrasound examinations, frequency settings (7.5 MHz), gain patterns (20 dB), and color of the Doppler scale were kept constant throughout the study. From the recorded videos, three different JPG images with maximum Doppler signals were obtained from the same region, using the Gom Player program (Gretech Corporation, Seoul, Korea). Then, the Image J program (National Institutes of Health, Millersville, USA), after initial calibration (1 cm = 25 pixels), was used to measure the area (cm2) of the colored pixels in each image. Then, the average of the areas obtained in the three images was calculated. Two-way ANOVA followed by the Fisher LSD test was used to evaluate the effects of transplant site and day on the area in pixels assessed by ultrasound. Data are presented as mean and standard error of the mean (± sem) with statistical significance defined as $P<0.05$. Analysis of the blood flow area around the transplanted ovarian fragments was evaluated daily from day 0 (pre-transplant) to day 15 (post-transplant), ending on days seven and fifteen, for removal of the implants on the left and right sides, respectively. Overall, the area of blood flow around the transplanted ovarian fragments was significantly greater ($P<0.05$) at the IA site compared to the IM site, after 7 (IA: 4.70 ± 0.33 vs. IM: 3.67 ± 0.33) and 15 (IA: 5.27 ± 0.21 vs. IM: 4.66 ± 0.22) days of transplant stay. The ultrasound evaluation carried out daily allowed us to observe that the blood flow area increased significantly in both sites evaluated, when comparing day 0 (pre-transplant) with the last day of evaluation, both 7 and 15 days after the transplant. These data showed that timely angiogenesis and effective perfusion of the microcirculation are essential for the survival and functional recovery of the grafted ovarian tissue, thus resulting in higher success rates (3). It is concluded that the IA site in goats presents itself as an effective model for ovarian tissue transplantation, as it is an easily accessible, minimally invasive site, has better monitoring when compared to more invasive sites (4), and has presented rates satisfactory results of tissue vascularization within the work performed.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Vaginitis in dogs: a retrospective study and clinical approach

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Vaginitis is a common reproductive condition, characterized by inflammation of the vaginal mucosa (1). The most prevalent clinical sign is the presence of vulvar purulent or mucopurulent discharge, although mucous secretion or even bloody discharge may occur (2). Distinct forms of classification are recognized: juvenile or adult vaginitis. The former affects prepubertal females, ranging from days to months with intermittent manifestation. Its etiology remains unclear and for its self-limiting nature, urgent treatment is not required. Chronic vaginitis in bitches older than 1 year of age most often is associated with identifiable abnormalities of the genitalia (35%) or urinary tract (26%) (3). Despite its prevalent occurrence within small animal theriogenology, vaginitis is a multifactorial disease that deserves an in-depth demographic survey, in order to improve its overall management in bitches. Hence, a retrospective study was performed, obtaining data over a 15-year time-frame (2008-2024) from the Gynecology and Obstetrics Section of the Veterinary Hospital of the School of Veterinary Medicine and Animal Science (University of Sao Paulo). A total of 45 vaginitis bitches were assisted during this period. Demographic data revealed a higher incidence among older castrated animals, with 46.6% of the bitches ageing 5 to 10 years old and 24.4% within 11 to 14 years old. The main clinical manifestations were abnormal vaginal discharge (greenish or yellow mucopurulent discharge), excessive licking of the genital area, redness, swelling of the vulva and abnormal vaginal odor. Of the total, 28.8% bitches had undergone gonadectomy, indicating a potential association between post-surgery hormonal changes and vaginitis. In fact, adult-onset vaginitis is more prevalent in spayed females, showing concurrent signs such as vulvar licking, pollakiuria, urinary incontinence, or systemic diseases like diabetes mellitus or immunocompromising conditions. However, comorbidities were observed in a small percentage of cases, including diseases such as ixodidiosis, leptospirosis, urinary incontinence, sarcoma, mastocytoma, and urinary tract infections. It is worth mentioning that 2.2% of the bitches had prior medical histories of antimicrobial therapy, including doxycycline, amoxicillin, cephalexin and ceftriaxone. This raises concerns about the potential negative impact of antibiotics on immune function, potentially predisposing individuals to vaginitis due to altered microbiota and weakened immune responses. Although diagnosis involves assessing clinical and behavioral signs, along with vaginoscopy, vaginal cytology alterations (indicative of a suppurative process) and microbiological culture, the presumptive diagnosis was achieved by history of discomfort and intense licking of the vulva, with no systemic involvement. The main differential diagnosis were uterine disorders, such as pyometra and endometritis, which was mainly achieved through ultrasonographic evaluation of the uterus. Treatment for vaginitis in bitches younger than 1 year of age is justifiably conservative, because the majority of such bitches (90%) recover with or without treatment (3). Hence, for therapeutic purposes, a protocol of vaginal acidification was employed, based on the administration of 500 mg – 1g ascorbic acid (vitamin C), TID, for 15 days. In refractory cases, culture of the vaginal discharge and antibiogram were performed, with specific antimicrobial use. Surgical correction was necessary whenever vaginal stenosis or septa were identified. In conclusion, vaginitis outcome is directly related to the resolution of other abnormalities as base cause. It presents multifaceted aspects influenced by age, hormonal status, surgical history, comorbidities, and medication use. Further research is warranted to elucidate its complex etiology and optimize therapeutic strategies for affected bitches.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Effect of *Vatairea macrocarpa lectin* on the *in vitro* development of sheep secondary follicles

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Among reproductive biotechniques, *in vitro* culture (IVC) of isolated ovarian follicles has stood out in terms of making viable oocytes available for other biotechniques, such as *in vitro* maturation (IVM) (1). However, the conditions of IVC are still not equivalent to the conditions of follicular development *in vivo*, which reduces the percentage of oocytes in metaphase II obtained from IVC. Lectins, like Vatairea macrocarpa lectin (VmL), which binds to lactose and galactose, common carbohydrates found in various glycoproteins present in most cell membranes (2) are potential substances for optimizing the growing environment is lectin. However, there are no studies on the effects of VmL on the *in vitro* culture of ovarian follicles. The aim of this study was to evaluate the effect of VmL on the morphology, development, glutathione (GSH) and ERO levels, mitochondrial activity, DNA fragmentation, and meiotic resumption of oocytes from sheep secondary follicles cultured *in vitro*. Isolated secondary follicles were cultured individually for 18 days in α-MEM supplemented with 3.0 mg/ml bovine serum albumin (BSA), 10 ng/ml insulin, 2 mM glutamine, 2 mM hypoxanthine, 5.5 µg/ml transferrin, 5.0 ng/ml selenium and 50 µg/ml ascorbic acid (control medium: α-MEM+) or in α-MEM+ with different concentrations of VmL (25 and 100 µg/ml). For inhibition of lectin activity, follicles were cultured in α-MEM+ supplemented with 25 µg/ml VML (control), 25 µg/ml denatured VmL, 25 µg/ml VmL associated with lactose (0.025 or 0.05 M). After culture, some of the oocytes underwent TUNEL assay and IVM. In addition, follicular morphology, GSH and mitochondrial activity concentration, DNA fragmentation and meiotic resumption rate were evaluated at the end of the culture. After 18 days of culture, there was no difference ($P>0.05$) among the treatments regarding morphology, and growth. After 12 and 18 days, antrum formation was greater ($P<0.05$) in treatments with VmL (89.9%) compared to control medium (59.1%). At the end of culture, the rates of fully grown oocytes and mitochondrial activity (≥110 µm) were similar ($P>0.05$) between control medium and 25 µg/mL VmL, and those were significantly higher than 100 µg/mL VmL. Furthermore, the intracellular GSH concentrations increased ($P < 0.05$) in oocytes from follicles cultured with 25 µg/ml VmL compared to the other treatments. After 18 days, there was a reduction ($P<0.05$) in the percentage of TUNEL-positive oocytes in 25 µg/ml VmL (8.6%) compared to α-MEM+ (35.1%). Moreover, VmL activity was significantly inhibited by denaturation and association with 0.05 M lactose, leading to a decrease ($P <0,05$) in the percentage of morphologically normal follicles (73.2 for denaturation and 72.9% for the association with 0.05 M lactose) and meiotic resumption compared to VmL alone (87.3% normal follicles). In addition, denaturation and culture with both 0.025 and 0.05 M lactose inhibited VmL activity, reducing antrum formation e GSH concentrations compared to VmL ($P<0,05$). Surprisingly, cultured with VmL associated with 0.025 M lactose increased ($P<0.05$) follicular diameter, and the rates of daily, as well as general growth compared to the other treatments. However, there was a decrease ($P<0.05$) in oocyte diameter induced by VmL denaturation and the culture with 0.05 M lactose. Lectins are proteins that act as important mediators in various biological processes, including cellular communication, cell adhesion, antioxidant activity and cell proliferation (3). In conclusion, 25 µg/ml VmL improves antrum formation, GSH levels and reduced DNA fragmentation in sheep secondary follicles cultured *in vitro*. It was possible to demonstrate that these effects were related to VmL activity.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Relationship between obesity, oocyte competence and doppler-velocimetry parameters

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Obesity is a multifaceted chronic condition characterized by an excessive accumulation of body fat, often leading to various metabolic, molecular, and hemodynamic disturbances. These alterations can profoundly affect reproductive health, including oocyte quality, in both humans and animals. Our study focused on investigating the impact of obesity on ovarian hemodynamics and oocyte quality in canines. Nineteen female dogs undergoing elective ovariosalpingohysterectomy (OSH) at private clinics in Fortaleza, Brazil, were enrolled in our research. The animals were divided into two groups based on their body condition score (BCS): the Control Group (CG), comprising healthy dogs with BCS ranging from 5 to 7, and the Obese Group (OG), consisting of obese dogs with BCS equal to or greater than 9. The Control Group (CG) comprises 15 female dogs, whereas the Obese Group (OG) consists of 4 female dogs. This stratification allowed for a comparative analysis of the effects of obesity on ovarian function. All subjects underwent Doppler triplex ultrasound evaluation to assess the hemodynamic parameters of the ovarian arteries, providing insights into blood flow dynamics within the ovaries. This procedure was performed on both ovaries. Following the OSH procedure, the ovaries were transported refrigerated at 4ºC in 50 mL Falcon tubes containing Phosphate Buffered Saline (PBS) to the Laboratory of Diagnostic Imaging Applied to Reproduction (LADIAR) for ovarian analysis. Oocyte retrieval, morphological evaluation, and Brilliant Cresyl Blue (BCB) staining were performed to assess oocyte quality and maturation status. Statistical analyses were conducted to compare the findings between the two groups, with results presented as mean values ± standard error of the mean. All statistical analyses were performed using R (version 4.0.2), R Foundation for Statistical Computing, Vienna, Austria. A Significance was determined at a 5% probability threshold ($p < 0.05$). Our results revealed significant differences between the CG and OG in systolic blood pressure parameters, indicating alterations in vascular function associated with obesity. Moreover, Doppler-velocimetric parameters showed notable differences in end-diastolic velocity (EDV) between the two groups, suggesting impaired ovarian perfusion in obese dogs. Furthermore, analysis of oocyte quality and BCB staining demonstrated distinct patterns between the CG and OG. The CG exhibited a higher proportion of grade 1 oocytes, indicative of superior quality, compared to the OG, which had a higher proportion of grade 3 oocytes, signifying compromised oocyte quality in obese dogs. Age serves as a notable risk factor for overweight and obesity, with a higher prevalence of obesity observed in older dogs, as supported by existing literature. Despite this, the mean age across the evaluated groups in our study did not exhibit significant variance, possibly due to the limited sample size and minimal age diversity among subjects. Notably, certain breeds are predisposed to obesity, yet in our study, a majority of dogs belonged to an indeterminate breed category, potentially altering perceptions regarding breed susceptibility to obesity. Significant differences in systolic blood pressure between obese and healthy dogs were observed, suggesting a potential link between obesity and hypertension, supported by previous research linking increased blood pressure with higher body condition scores. Doppler evaluation revealed challenges in obese dogs, with slight differences in diastolic velocity observed between groups, necessitating further investigation with a larger sample size. Additionally, differences in oocyte quality between groups were noted, indicating a negative impact of obesity on oocyte grading, consistent with findings in other species. These results suggest potential subfertility in obese dogs, warranting further exploration into the role of body condition score in managing reproductive biotechnologies for canines.

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FEMALE REPRODUCTIVE BIOLOGY

NITRITE LEVELS IN CULTURE MEDIUM FROM VITRIFIED BOVINE OVARIAN TISSUE IN THE PRESENCE OF *PUNICA GRANATUM* L.

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Cryopreservation followed by transplantation is the most viable technique for preserving and restoring women fertility after oncological treatments, since woman may suffer from premature ovarian insufficiency. Cryopreservation is a technique for long-term preservation, which can be carried out slowly by programmable freezer or quickly by vitrification. Vitrification is an effective technique that presents satisfactory results. However, it is necessary to add high concentrations of cryoprotectants, which may cause cytotoxicity to vitrified tissues, leading to morphological, biochemical, and/or molecular damages. Thus, the present study aimed to evaluate the levels of nitrite (NO2-), an important biochemical marker, in the culture medium of tissues cultivated after vitrification in different concentrations of the ethanolic extract of Punica granatum L. The ovarian cortex fragments were subjected to the standard vitrification solution, which was composed of alpha-MEM; 0.25M Sucrose; 10% Dimethylsulfoxide and 10% Fetal Bovine Serum. Furthermore, the standard solution was added of 10, 50 and 100 µg/mL of the ethanolic extract of Punica granatum L. For all vitrification solutions, the exposure time was 5 minutes. Vitrification of ovarian fragments was performed on a solid surface using a metal plate in liquid nitrogen. The fragments were then stored in nitrogen for 5 days. At the end of the storage period, all the samples were heated and incubated *in vitro* for 24 hours and the culture medium collected for later analysis. The determination of NO2- levels was carried out in duplicate using the Griess reaction. 100 µL of the culture medium and 100 µL of Griess solution (1% sulfonylamide in 5% H3PO4; 0.1% NEED/ distilled water/ 1:1:1:1) were used. The standard curve was produced from the serial dilution of a sodium nitrite (NaNO2) solution. Readings were obtained at A540 nm, and values were expressed in µM of NO2-. As a result, it was possible to observe that nitrite levels do not differing significantly among treatments. Nitrite is one of the products of the oxidative metabolism of nitric oxide and its production could indicate oocyte maturation and embryo quality in human (1). In addition, nitrite may act as a dose-dependent regulator of steroidogenesis in bovine granulosa cells (2). Therefore, considering that no changes in nitrite production were observed, its dosage could it be considered an important marker of the cryoprotective effects of the ethanolic extract of Punica granatum L. during pre-antral folliculogenesis.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Punica granatum L. reduces thiol content in bovine ovarian tissue cultured *in vitro*

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Reproductive biotechnology have been means for the recovery of pre-antral ovarian follicles in order to obtain competent oocytes and genetic improvement through *in vitro* culture, this technique being relevant to preserve reproductive capacity. However, the *in vitro* environment can generate oxidative stress in the cultured tissue due to the influence of temperature and light variations, thus increasing the production of reactive oxygen species, resulting in cellular damage due to the imbalance of ROS caused by redox imbalance (1). To minimize the effects of oxidative stress, plants and extracts with antioxidant effects have been studied. Thus, the aim of the present study was to investigate the antioxidant potential of the ethanolic extract produced from the peel of pomegranate fruit (Punica granatum L.), on thiol levels in bovine ovarian tissue cultured *in vitro*. The peel of Punica granatum L. fruit was removed from fresh and ripe fruits, originating from commercial cultivation, and taken to the Laboratory of Natural Products Chemistry (LQPN) of the State University of Ceará, being submerged in ethanol (PA 99.8%) for 5 days. After that, the liquid resulting from the extraction of the peel components was directed to a rotary evaporator at 50°C, where the solvent was completely evaporated, and only the superconcentrated extract was removed for further analysis. Finally, this extract was lyophilized and kept at a temperature equal to or lower than 10°C, and the powder was diluted in ultrapure water, using concentrations of 10, 50, and 100 µg/mL. The present study was approved by the Ethics Committee on Animal Use (CEUA) of the State University of Ceará (UECE) under protocol no. 09326942/2021. For the culture experiment, ovarian cortex from cows (n=24) without a defined breed standard were fragmented and subsequently cultured for 6 days in αMEM medium added with ITS (10 µg/mL insulin, 5.5 µg/mL transferrin, and 5 ng/mL selenium), BSA (1.25 mg/mL), Glutamine, and Hypoxanthine (αMEM+), in the absence (cultured control) or presence of different concentrations (10, 50, and 100 µg/mL) of ethanolic extract of Punica granatum L. (EE-cPG). The culture was carried out in an incubator at 38.5°C and a humidified atmosphere with 5% CO2. Analysis of variance (ANOVA) with Tukey or Mann-Whitney post-hoc tests was used, respecting the normality test, with a P value <0.05 considered statistically significant. As a result, the concentration of 10 µg/mL of EE-cPG significantly reduced the thiol content when compared to the cultivated control and fresh control, while the concentrations of 50 and 100 µg/mL maintained similar levels. In view of that, it is suggested that concentrations of 50 and 100 µg/mL of the ethanolic extract of Punica granatum L. are efficient for maintaining thiol levels after *in vitro* culture.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Wharton's Jelly mesenchymal stem cells stimulate follicular development and possess the ability to differentiate into germ cells

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In recent years, several efforts have been made to establish an effective method for *in vitro* culture of fresh or vitrified ovarian tissue. In this context, mesenchymal stem cells (MSCs) emerge as an excellent option due to their multipotent nature, high self-renewal capacity, and differentiation potential [1]. In this study, we evaluated, for the first time, the effect of co-culturing fresh or vitrified sheep ovarian tissue with Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) for 14 days. For this purpose, we used five pairs of ovaries from adult sheep (N=10), with each pair generating eighteen fragments distributed into fresh (n=9) and vitrified (n=9) groups. Three fresh fragments were immediately fixed as fresh controls, while the other six were cultured *in vitro* with or without WJ-MSCs for 14 days. Three of the vitrified fragments were fixed immediately after warming (vitrified controls), and the other six were cultured *in vitro* under the same conditions described for the fresh group. All fragments underwent morphological evaluation and assessment of follicular development using classical histology, senescence via Sudan Black B staining, and apoptosis analysis through RT-qPCR targeting BAX and BCL2 genes. Statistical analysis was conducted using Sigma Plot 11.0, with mean comparisons performed using one-way ANOVA followed by Fisher LSD post hoc test, considering values significant when $p < 0.05$. The results indicated that the follicular morphology of fragments cultured in fresh (93.3 ± 2.8%) and vitrified (80.7 ± 3.7%) groups with WJ-MSCs remained similar to fresh (100.0 ± 0.0%) and vitrified (86.7 ± 4.9%) controls, respectively. Although all cultured treatments showed follicular development, oocyte extrusion was observed only in groups co-cultured with WJ-MSCs. These results may be attributed to the action of various factors such as growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), Kit ligand (KL), leukemia inhibitory factor (LIF), and cyclic adenosine monophosphate (cAMP), which play roles in the maintenance and promotion of follicular development [2]. Regarding senescence, culturing vitrified ovarian tissue in the presence of WJ-MSCs reduced the presence of senescent cells, suggesting a beneficial effect of WJ-MSCs on cell proliferation and senescence inhibition, possibly mediated by the secretion of fibroblast growth factors (FGFs) by WJ-MSCs [3]. The BAX results showed increased expression in all treatments compared to the control, while BCL2 remained similar to the control, except in fresh culture in the absence of WJ-MSCs, which showed a significant reduction. MSCs include anti-apoptotic mechanisms that positively regulate DNA repair and negatively regulate death pathways [4]. Additionally, a very interesting finding was observed during the study, namely the formation of cellular spheroids with a germinative profile in all treatments co-cultured with WJ-MSCs. Upon evaluation, it was found that these spheroids exhibited positive markers for germ cells (DDX4) and cell proliferation (PCNA), indicating the intrinsic capacity of WJ-MSCs to differentiate into oocyte-like cells. This phenomenon may be attributed to the germline cell memory of WJ-MSCs combined with the culture environment [5]. In conclusion, our results demonstrate that WJ-MSCs have a positive impact on survival and follicular development, as well as showing the capacity for differentiation into germline lineages, making them a promising source for future investigations related to *in vitro* oogenesis.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Impact of energy supplementation on the reproduction of crossbred Santa Inês and Morada Nova sheep in rotational grazing system

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Several strategies have been implemented to minimize the impact of food scarcity on animal reproduction, since this is fundamental to ensuring a successful production. Among these strategies, we can highlight the provision of food supplementation during the reproductive period, including the period prior to fertilization and during animal gestation (flushing food), which allows for a significant increase in the reproductive parameters of ewes, due to increased ovulation rates and, therefore, twin births (1). Therefore, the aim of this study was to test the efficiency of energetic supplementation provided during the reproductive season for breeds of yearling sheep raised on a rotational grazing system. The experiment was conducted at the Experimental Farm of the Vale do Acaraú State University using a total of 96 adult, non-pregnant mixed-breed ewes of different ages. 48 Santa Inês sheep and 48 Morada Nova sheep were randomly divided into two groups, non-supplemented and supplemented, for each breed. The experimental diet fed to the sheep in the supplemented groups consisted of ground Tifton 85 hay (70%), 94.67% ground corn, 5.04% soybean meal and 0.29% limestone. Feed supplementation was provided two weeks before and throughout the reproductive period, which lasted 45 days. Reproductive performance was assessed using the ewe's body weight at birth, birth weight (kg) and pounds of lamb produced per ewe (kg). A completely randomized design was used in a 2 x 2 factorial arrangement, with two breeds (Santa Inês and Morada Nova) and two treatments (supplemented and non-supplemented). The data was analyzed using SAS version 9.1 (2003). The results showed that none of the variables evaluated were affected by the experimental diet, where similar results on the ewe's body weight at birth were reported by previous researches, in studies with wool ewes (2). It is believed that the animals' adaptability to the semi-arid environment and the ewes' good body condition contributed to minimizing the effects of supplementation on reproductive parameters. Therefore, the practice of energy supplementation provided during the breeding season did not influence the productivity of mixed-breed ewes of the Santa Inês and Morada Nova genetic groups, and can be completely dispensed with for ewes of these genotypes when they are in satisfactory body condition.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Effect of L-carnitine on the growth of *in vitro* cultured bovine secondary follicles

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The *in vitro* culture of isolated secondary follicles aims to obtain mature oocytes suitable for *in vitro* fertilization. However, factors such as high oxygen tension associated with light interference, temperature, and the loss of natural antioxidant protection after follicular isolation may favor oxidative stress and decrease cell antioxidant protection, contributing to the low efficiency of *in vitro* culture systems (AGARWAL et al., 2018). Thus, to minimize the deleterious effects of free radicals and to promote an increase in follicle growth, various natural molecules, recognized for their antioxidant effects, have been added to culture media. Among these molecules, one that has gained prominence is L-carnitine. However, its effects during *in vitro* culture of secondary follicles in the bovine species have not yet been elucidated. The aim of this study was to assess the effects of different concentrations of L-carnitine on *in vitro* growth of bovine secondary follicles after 18 days of culture. For this, secondary follicles (~0.2mm) were mechanically isolated from ovaries and cultured in an incubator with 5% CO2 at 38.5ºC for 18 days, in TCM-199 supplemented with BSA (1.25 mg/mL), ITS (insulin 10 µg/mL, transferrin 5.5 µg/mL, and selenium 10 µg/mL), glutamine (2 mM), hypoxanthine (2 mM), ascorbic acid (50 µg/mL) under mineral oil (control medium denominated TCM-199+). The follicles were thus cultured in TCM-199+ alone or supplemented with different concentrations of L-carnitine (10, 50, or 100 µM). The follicular diameter data were analyzed using the Kruskal-Wallis test followed by the Dunn's multiple comparisons test. Differences were considered significant when $P < 0.05$. As a result, it was observed that the cultured follicles showed progressive growth until the sixth day of culture in all treatments. However, from the 12th day of culture, significant growth was not observed. On the sixth day of culture, follicles cultured with 100 µM acetyl-L-carnitine showed a significantly larger diameter compared to those cultured in control medium. However, no significant differences were observed when compared to follicles cultured with other concentration of L-carnitine. In conclusion, the addition of 100 µM acetyl-L-carnitine in the culture medium increases the diameter of bovine secondary follicles in the first 6 days of culture.

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X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Effects of resveratrol-loaded polymeric nanoparticles on the levels of thiol and activity of superoxide dismutase, catalase and glutathione peroxidase in *in vitro* cultured bovine ovarian tissues

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Increasing the availability of oocytes suitable for *in vitro* embryo production is one of the main goals of *in vitro* culture of bovine preantral ovarian follicles. However, some obstacles, like oxidative stress, compromise the *in vitro* development of these early follicles. Resveratrol is a relevant alternative to supplement culture medium due to its antioxidant characteristics. However, a significant part of this antioxidant potential is lost due to low absorption during culture. Therefore, incorporating resveratrol into polymeric nanoparticles is a valuable alternative to increase the availability of this compound. It is also important to note that enzymes like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) have a key role in the cellular defense against oxidative stress. This study aimed to evaluate the effects of different concentrations of resveratrol-loaded polymeric nanoparticles and free resveratrol on the levels of thiol and activity of SOD, CAT and GPX in *in vitro* cultured bovine ovarian tissues. For this purpose, ovarian tissue from 10 ovaries (five cows) were cultured for 6 days in the presence of different concentrations of resveratrol-loaded polymeric nanoparticles (0.2, 2.0 and 20.0 µM) and free resveratrol (20 µM). Culture conditions were 38.5°C and 5% CO2 in air. For analyzing the activity of antioxidant enzymes, tissue samples from different treatments were homogenized in potassium phosphate buffer, pH 7.5. The homogenates were centrifuged at 1500 g for 10 min at 4°C, and the supernatant was collected for use in spectrophotometric assays using quartz cuvettes. Data were expressed as the mean ± SEM of enzyme unit per milligram of protein (U/mg of protein). Protein concentration was determined using the Bradford method. Total thiol content was determined using 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) as an index of reduced thiol. SOD activity was measured by adrenaline autooxidation inhibition. The CAT activity was measured by hydrogen peroxide (H2O2) consumption as a substrate at 240 nm. The GPX activity was measured by NADPH oxidation. Statistical analysis was performed by GraphPad Prism software (5.0). The results showed that tissues cultured with resveratrol alone or resveratrol-loaded polymeric nanoparticles, at all concentrations tested, had reduced SOD activity when compared to tissues cultured in control medium. No differences were seen among activity of SOD in tissues cultured with different concentrations of resveratrol. Regarding CAT, the free resveratrol (20 µM) increased the activity of this enzyme in cultured ovarian tissues, when compared to those cultured in control medium. The resveratrol-loaded polymeric nanoparticles did not influence the activity of this enzyme. In addition, free resveratrol (20 µM) increased the activity of GPX, while resveratrol-loaded polymeric nanoparticles (0.2, 2.0 µM) reduced the activity of this enzyme in cultured tissues. resveratrol-loaded polymeric nanoparticles (0.2µM) also reduced the levels of thiol in cultured tissues. In conclusion, free resveratrol reduces the activity of SOD and increase the activity of CAT and GPX, while the resveratrol-loaded polymeric nanoparticles (0.2µM) reduces the activity of SOD, GPX and thiol levels.

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X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Use of mesenchymal stem cells in mares with chronic endometritis

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Endometritis is a local inflammatory process, reaching mainly the most superficial layers of the uterus in response to foreign agents such as seminal plasma, sperm, semen proteins and bacteria. The inability to eliminate intrauterine fluid and inflammation leads to reproductive inefficiency and a significant economic loss to the equine industry. Mesenchymal stem cells (MSCs) have an anti-inflammatory and immunomodulatory properties and the potential to treat inflammatory disorders. The objectives of this study were to evaluate the ability of MSCs to modulate the inflammatory response in susceptible mares. Eleven mares aged 5-19 years old were classified as susceptible to persistent endometritis based upon their history of at least 4 negatives embryo transfer flushes. The mares were used in a cross-over design with each mare being control of itself. To evaluate the effects of intrauterine administration of allogeneic MSCs on the pregnancy, treatment was tried 1or 2 times (in the mares that did not get pregnant in the first trial). Mesenchymal stem cells were obtained through collection of adipose tissue from a healthy equine donor. The tissues were then washed in DMPBS solution and subjected to enzymatic digestion in collagenase and hyaluronidase solution for 30 minutes at 37.5°C, where mononucleated cells were isolated and added to culture with DMEN medium (SIGMA D5523) at 37.5° C and controlled atmosphere at 5% CO2 for 7 days, with medium change every 48 hours. After it was cultivated until reached 90% confluence and then cryopreserved in liquid nitrogen. For use in direct treatment, cells were thawed in a water bath at 37° for 20 seconds. Afterwards, it was inserted into a defrosting medium, with centrifugation at 2000 rpm for 3 minutes. The supernatant was removed by pipettor, and the washing medium with PBS was added and centrifuged at 2000rpm for 3min, repeating this procedure three times. At the end, the content was added and homogenized to the specific transport medium, with 1mL of medium added for each million cells. A uterine lavage was performed using 1 liter of glucose 5% and then 10 mL of MSCs was infused in the uterus of the mares. From the eleven mares, 9 (81,8%) got pregnant. From those 9 mares, 4 got pregnant after the first treatment and the other 5, got pregnant at the second treatment, one of those got an embryo and a pregnancy after this flush. Although it is a preliminary result, we conclude that MSCs infusion in endometritis mares can increase pregnancy rate and can be a useful therapy of uterine diseases in susceptible problem mares.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

STUDY OF KEY FACTORS IN THE REPRODUCTIVE AND PRODUCTIVE PERFORMANCE OF HAIR SHEEP IN THE SEMI-ARID REGION OF NORTHEAST BRAZIL

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Reproductive efficiency and herd growth play fundamental roles in the profitability of animal production, wherein hair sheep demonstrate superior productivity, attributed to their higher ovulation and reproduction capacity. This success, in turn, is closely linked to fertility at birth and the number of lambs born (1). Therefore, the present study aimed to analyze the determining factors of ewe weight at parturition, as well as lamb birth weight and herd productivity, expressed in kilograms of lamb produced per ewe after parturition. The experiment was conducted at the Experimental Farm of the State University of Vale do Acaraú, located in the Northeastern semi-arid region, characterized by an average annual temperature of 32°C. Ninety-six pregnant and multiparous ewes were used, equally divided into two experimental groups: 48 crossbred Santa Inês sheep and 48 Morada Nova genetic group sheep. Throughout the experiment, all ewes were kept on rotational grazing, feeding on pastures planted with Tifton 85. The breeding season lasted 45 days, involving five rams, two of Santa Inês breed and three of Morada Nova breed. Ewes were weighed in the last week of gestation, and throughout the reproductive period, Body Condition Score (BCS) was assessed weekly, following literature guidelines (2). Lamb weights at birth were recorded within 12 hours after parturition, and the variable of kilograms of lamb produced per ewe after parturition was calculated considering the total weight of the offspring. The experiment followed a completely randomized design, and data analysis was performed using SAS software (3), with analysis of variance to examine the effects of genetic group, type of birth, and age of dams, as well as the birth weight of lambs. Results indicated that lamb birth weight was significantly influenced by age and breed, with higher post-partum weights observed among more mature specimens. These observations can be explained by the fact that young females have not yet reached their full body maturity, resulting in lower birth weights compared to adult females (4). Regarding the genetic group, crossbred Santa Inês sheep exhibited higher birth weights compared to those of the Morada Nova breed group. Additionally, lamb birth weight was significantly influenced ($p < 0.05$) by ewe weight at parturition and birth method. However, the genetic group had no significant effect on lamb birth weight; however, these results differ from those found by other authors[4]. The birth method had a significant impact on offspring birth weight, as lambs born from single births had higher weights compared to those born from twin births, due to the absence of intrauterine space competition. Regarding the variable of kilograms of lamb produced per ewe after parturition, significant effects ($p < 0.05$) of the BCS class of females, genetic group, and type of birth (single or twin) were observed; however, age and weight of ewes at parturition did not affect lamb productivity in kilograms. Therefore, it is concluded that ewe body weight at parturition is influenced by genetic group and age at parturition, given that lamb birth weight can be influenced by birth type and ewe weight. Body condition score, breed group, and birth type also influenced lamb productivity per ewe after parturition.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Markers of oxidative stress in the colostrum of mares in the peri-partum period

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The average gestation length in mares ranges from 320 to 388 days and can be influenced by several factors intrinsic and extrinsic to the female. The birth of a viable foal depends on the coordination of several endocrine events involved in the maintenance of pregnancy, fetal maturation and maternal preparation for birth. When such processes deviate from the physiological standard, a high risk of pregnancy loss and/or neonatal death becomes present, which leads to economic losses. Therefore, we can consider that predicting the moment of the birth is an important tool that aims to guarantee maternal health and the viability of the newborn. However, the methods currently available for this purpose are still not very precise. With the objective to overcome this problem, we aimed to evaluate colostral oxidative stress parameters as possible markers of the proximity of parturition in mares, considering that according to recent studies in bovine females, antioxidants and reactive oxygen species (ROS), increase in the pregnant mother in the last few weeks before giving birth, and are also found in breast secretion. Therefore, 3 mares were evaluated from the 9 days before parturition, in which colostrum samples were collected daily until the day of parturition, after delivery of the product. To analyze the oxidative profile, all colostrum samples were centrifuged at 3,500 rpm for 10 minutes to obtain milk serum and stored in an eppendorf tube at -20 °C until analysis. By measuring metabolites of malondialdehyde (MDA), Ferric Reducing Antioxidant Power (FRAP), catalase (CAT), superoxide dismutase (SOD) and carbonyl protein (CP), it was possible to evaluate the biochemical changes related to the colostral oxidative stress state. The absorbance of each test was read by the spectrophotometer at a wavelength of 374 nm for CAT, 320 nm for SOD, 593 nm for FRAP, 535 nm for MDA and 370 nm for CP. The results were statistically analyzed using the SPSS 21 software, where the data were subjected to descriptive analysis and the results expressed as mean and standard deviation. The quantitative variables were subjected to the Shapiro-Wilk and Levene tests to verify the normality of errors and homogeneity of variances, respectively, compared using the Friedman test. All statistical tests were performed with a 5% probability of error. In the individual assessment, the CP variable showed third-degree polynomial behavior with R^2 varying between 0.8578 and 0.9222, showing a value with a high degree of confidence. Furthermore, it was possible to observe through the descriptive values that the CP on day 9 before birth (D-9) had an average of 66.55, and varied without statistical difference until D-4, from that day onwards the beginning of a gradual increase, in which it presented an average ± standard deviation: 81.67 ± 27.27 in D4; 118.67 ± 36.97 in D-3; 94.51 ± 34.22 in D-2; 161.44 in D-1; and 427.53 ± 28.15 in D0. The abrupt increase in the day of birth and the day before birth from D-4, significantly showing a statistical difference between these values ($p<0.05$). We can also observed that the CP concentration was above 250 nmol/ml for all animals on the day of birth, and that this value increased abruptly on the day before birth. Thus, about the results presented for carbonyl protein, it appears that it can be a potential marker of proximity to birth. However, more studies need to be conducted to confirm the results obtained, requiring a larger sample for this purpose.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

THE APPLICABILITY OF RESVERATROL NANOPARTICLES IN THE EXTRACELLULAR MATRIX IN THE *IN VITRO* CULTURE OF BOVINE OVARIAN TISSUE

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In vitro culture of ovarian follicles makes it possible to study folliculogenesis. It also provides many viable oocytes for in vitro fertilization and cloning biotechniques. However, the technique faces some limitations, such as oxidative stress, which significantly affects ovarian tissue and the integrity of the extracellular matrix (ECM). The ECM is an essential component for the survival of granulosa cells, stimulating differentiation and cell surveillance. It plays an active and essential role in folliculogenesis and the structural support of ovarian follicles. In this sense, it is important to evaluate the positive effects of resveratrol, a natural polyphenol with anti-inflammatory and antioxidant properties, during the in vitro culture of bovine ovarian follicles. The ovaries (n = 10) of 5 adult cyclic cows were collected from a local slaughterhouse. In the laboratory, the cortex of each ovary was fragmented and subjected to in vitro culture in 0.5 ml of control medium (α-MEM+) alone or supplemented with different concentrations of free resveratrol and nanoparticles (NPRSV 0.2 µM, NPRSV 2µM, NPRSV 20 µM, NPBR 20 µM and RSV 20 µM). The medium was changed every two days out of six and 60% (300µl) of the culture medium was replaced with fresh medium. At the end of cultivation, the samples were used to evaluate the collagen fibers of the extracellular matrix. After processing for histology, the slides with ovarian sections (6 µm) were deparaffinized in xylene and incubated in Sirius Red solution (0.1%) for 1 hour at room temperature following the methodology described by Rittié (2017) with modifications. For each treatment, the percentage of the area occupied by collagen fibers in ten different fields will be measured, and the images will be analyzed using Image J software (version 1.51p, 2017) at 400x magnification. Statistical analysis will be carried out using GraphPad Prism software (5.0). The results show a significant reduction in the percentage of the area occupied by collagen fibers in all treatments after in vitro culture, compared to the fresh control. However, when the treated groups were compared with each other at the end of the cultivation period, it was found that supplementing the medium with 0.2 or 2 uM of resveratrol-loaded nanoparticles in the medium maintained higher levels of collagen fibers than those observed in the cultivated control group and the treatments of 20 uM resveratrol-loaded nanoparticle, white nanoparticle and 20 uM resveratrol. According to the results, even with the reduction in collagen fibers compared to the fresh control, the use of nanoparticles with resveratrol at a concentration of 0.2 or 2 uM provided an increase in collagen levels compared to the cultured control group, so it can be concluded that encapsulated resveratrol is an effective alternative during the ovarian tissue culture period, with the ability to preserve tissue collagen while maintaining the structure of the extracellular matrix.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Equine platelet-rich plasma: comparative analysis of treatment methods for platelet measurement

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Given the economic and social importance of equine breeding, this area has been in constant evolution, and the use of new therapies that help treatment conditions that affect these animals are essential. Endometritis is one of the most important diseases that affect reproductive system of horses, being considered one of the biggest causes of subfertility and infertility in mares. Platelet-rich plasma (PRP), a blood product obtained through centrifugation and rich in growth factors, has emerged as a promising treatment for numerous pathologies. Growth factors are stored in large quantities in platelets and are responsible for regulating cellular metabolism through signaling pathways of a complex of cell surface receptors. This process increases transcription factors and protein production, stimulating cell proliferation and differentiation, in addition to boosting the production of the extracellular matrix. This, together with the stimulation of neovascularization, assists in the tissue repair process. The PRP treatment acts as an immunomodulator of the inflammatory response, improving fertility of barren mares with persistent mating-induced endometritis (PMIE). It is important to note that treatment using PRP generally requires multiple applications and the preparation of the product, ensuring its precise concentration is very important. Therefore, the present work aimed to analyze the impacts of different manual methods with the addition of dyes on the platelet count in the PRP of horses. For this purpose, five healthy horses were used, and the blood was collected via venipuncture. Samples were processed to obtain PRP using a double centrifugation protocol. Manual platelet counting was performed using three methods: the Brecher method, the G&S method with HAMA methylene blue and the modified Rees-Hecker method. Brecher's method is based on platelet counting, in which 2 ml of 1% ammonium oxalate and 20 µL of sample were added to a test tube under analysis. After homogenization of the solution, the Neubauer chamber was filled and incubated in a humid chamber for 10 minutes, counting was carried out after this period under a bright field microscope, with a 40x objective. The G&S methodology, 425 µl of 1% ammonium oxalate and 20 microliters of the sample under analysis are used. After mixing slowly for 20 seconds, the sample is kept for 18 minutes of incubation at room temperature and then 15 µl of HAMA methylene blue is added. The Neubauer chamber is filled after incubation for 2 minutes in a humid chamber. The count is carried out under a bright field microscope, using a 40× objective, with the condenser completely lowered and the diaphragm almost completely closed. In this method, platelets must be visualized and distinguished from other elements due to their intense blue color, refringence and typical shape. For the third protocol tested, the Rees-Hecker method, 450 µl of 1% ammonium oxalate and 20 microliters of the sample under analysis are added. After incubation for 15 minutes at room temperature, 20 µl of Rees liquid is added. The Neubauer chamber is filled immediately after incubation for 10 minutes in a humid chamber. Counting takes place under a bright field microscope, using a 40x objective. In all samples, counting in the Neubauer chamber was performed in five squares of the central lattice (outer corners and center) on each side of the chamber, totaling ten counting areas. After summing, the final value was multiplied by 2500, with the number obtained equivalent to the total number of platelets per/µL of blood. The results were statistically evaluated using one-way ANOVA and Student's t test, with correlation performed using Pearson's correlation test. There was no significant difference between the counting methods evaluated; however the correlation between the Brecher method and the G&S method was positive and between the Brecher method and the modified Rees-Hecker method was slightly negative. This latter result suggests a lack of association between the platelet values observed in the protocols. Therefore, it was concluded that the G&S method exhibits a similar degree of reliability to the Brecher method, facilitating the visualization of platelets in equine PRP. This is due to the correlation between protocols. Further evaluation will be necessary to confirm the equivalent reliability of the modified Rees-Hecker method, as well as its standardization.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Toxic effects of chromomycin A5 on ovine ovarian tissue cultured *in vitro*

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Premature ovarian failure mainly occurs due to the toxicity of chemotherapy drugs, which can lead to a complete depletion of the ovarian reserve of preantral follicles, compromising the well-being of women undergoing cancer treatment (1). In this manner, efforts have been made to discover new drugs with minimal side effects, especially concerning reproductive ovarian function. The marine environment has been the focus of investigations for new compounds, and chromomycins, metabolites isolated from marine bacteria Streptomyces, exhibiting potent antiproliferative activity in various tumor cell lines (2). Thus, this study aimed to investigate the toxicity of chromomycin A5 (CA5) on the *in vitro* development of ovine preantral follicles and ovarian stromal tissue. To this end, sheep ovaries were collected from a local slaughterhouse and slices of the ovarian cortex were removed and randomly assigned to *in vitro* culture (IVC) in six experimental conditions: non-cultured tissue (NC), *in vitro* cultured for 1 or 6 days in MEM supplemented medium (MEM+); IVC with 0.3 µg/mL doxorubicin (DOX) or IVC with 100, 200 or 300 nM CA5 (CA5100, CA5200 and CA5300). After one (D1) or six days (D6) of IVC, the tissue was fixed and processed for classical histology (PAS-HE and Sudan black SB) to evaluate follicular morphology and development, stromal density, and lipofuscin accumulation. Immunohistochemistry for PCNA and TUNEL to verify proliferation and DNA fragmentation was performed. Data are presented as percentages (mean ± SEM), and differences were considered significant when $P < 0.05$. All statistical analyses were performed with SPSS software. A total of 2,640 follicles were analyzed by classical histology and demonstrated that exposure of ovine ovarian tissue to all doses of CA5 after 6 days of IVC resulted in progressive loss of preantral follicles. At D1 of culture, CA5300 (57.9±4.6) demonstrated the lowest rate of follicular morphology ($P<0.05$; NC= 100; MEM+= 99.2±0.5; DOX=74.3±3.8; CA5100=86.3±4.9; CA5200=74.2±5.8). At D6, DOX (58.3±4.2), CA5200 (40.4±5.4), and CA5300 (36.3±2.3) significantly reduced survival in all follicular categories when compared to MEM+ (80.4±8.30). Regarding the follicular development, on D6 CA5200 and CA5300 showed the lowest percentage of developing follicles and cellular stromal density compared to MEM+ ($P<0.05$). At D6, DOX and all CA5 concentrations demonstrated a high percentage of senescent cells and DNA fragmentation compared to MEM+. Moreover, CA5300 was found to be strongly toxic to ovarian tissue and significantly reduced the cell proliferation rate compared to MEM+. In conclusion, CA5 exerts a toxic effect observed by decreasing follicular survival and development, cell proliferation, and inducing senescence, similar to DOX. We suggest that further studies need to be performed to identify not only the mechanism of action of CA5 but also its effect on preantral follicles in more advanced stages of development, aiming for a deeper investigation into the reproductive function of females.

Keywords: Preantral follicles; ovarian failure; citotoxicity.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Bibliometric analysis in animal reproduction over the last 10 years using R software

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Introduction: In the last 10 years, we have experienced an increase in scientific publications and the use of filter tools to better target initial studies is fundamental. However, just the filters available on digital platforms for researching scientific works are not sufficient to identify trends and gaps, requiring statistical treatment using software that is capable of identifying predominant bibliometric markers. Materials e methods: Considering scientific publications from the last decade, two bibliometric treatments were carried out in the area of animal and human reproduction using the term "artificial ovary" as a keyword for searching on the Web of Science and Scopus platforms with the aim of identifying the main trends in the area. The first treatment consisted of a bibliometric analysis of articles from the last 10 years using the bibliometrix package in the R software and using the two databases simultaneously and the second treatment a bibliometric analysis of the last 5 years. The biomarkers for the first treatment were: publications per year in the area, most productive researchers, most reported articles, countries with the most productive researchers in the area, most relevant newspapers and most used keywords. 1860 documents and 790 Scopus documents were initially filtered on the Web of Science platforms. In an initial treatment using the R software, 286 duplicate documents were excluded, resulting in a database of 2364 documents. Results e discussion: The results showed that there was an increase in publications between 2013 (160 documents) and 2022 (324 documents), however the year 2023 (277 documents) showed a slight reduction in productivity of approximately 8%. Among the researchers with the highest production, Wang stands out with 53 documents, with the years 2021 and 2022 being the years with the highest production. Amorim and Dolmans are the most cited researchers and the journals Theriogenology and Journal Dairy of Science have the largest number of relevant articles, 128 and 57, respectively, both journals have a local impact factor of 23. China, USA and Brazil are the countries with the largest number of researchers and published works, with the University of Florida (116 documents in 2023) and the University of São Paulo (96 documents in 2023) with the greatest growth in publications in the 10 years of research. Among the key words: ovary, progesterone, pregnancy and ovarian cancer are among the most used and the words used in female titles were the most used. When comparing analyzes of 10 years with 5 years, there are no important differences. We conclude that research groups remain active for more than 10 years and we suggest analyzes in different temporal spaces to identify changes in research groups over time.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Does chromosomal instability in triploid Nile tilapia affect its reproductive status?

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A commonly reported problem for Nile tilapia is early sexual maturity, which can cause overcrowding, extend fattening time, increase weight variation in the batch and reduce the proportion of animals that reach commercial weight. Therefore, reproductive control is one of the biggest challenges in tilapia production and triploidy was developed as an alternative to sterilization. Triploid individuals are desired in aquaculture due to the inability of homologous chromosomes to pair equally in gametogenesis, rendering the organism theoretically sterile. However, in general, polyploids present chromosomal instability, and it is not known to what extent the sterility of these individuals can be affected. The aim of this study was to evaluate the gonadal status of male and female tilapia hatched from eggs subjected or not to heat shock for triploid induction. Nile tilapia oocytes were fertilized (1,476 oocytes), half of the eggs were subjected to a four-minute shock in 41°C water four minutes after fertilization, and the other half were not (Control group). The eggs were incubated (at 27°) and 160 larvae from the treated group hatched and survived after yolk sac absorption. The determination of ploidy was performed by flow cytometry at 85th (juveniles) and 301st (adults) days of age post yolk sac absorption. When the animals were 127 and 275 days old after yolk sac absorption, seminal fluid was collected to determine the sperm concentration, motility and vigor of the individuals' semen. Ten days after semen analysis, an attempt was made to compare the reproductive performance of animals (diploids and triploids based on the first chromosomal analysis) by induced spawning using diploid females with diploid and triploid males. However, the triploid males did not produce enough semen for fertilization, and only eggs from the crossing of females and males from the control group (diploids) obtained satisfactory results. At the end of the experiment, the animals were euthanized, followed by evisceration, with separation and weighing of the gonads for histological and morphometric analyzes and calculation of the gonadosomatic index. Each individual constituted an experimental unit. Comparisons were made between the groups: control, subjected to non-triploid shock, lost triploid status, and confirmed triploid (based on the second chromosomal analysis). At the time of the first cytometry analysis there were 73 surviving juveniles from the treated group, and only 14 were confirmed triploid. However, at the analysis of adult ploidy, one out of 8 surviving adult tilapias from the 14 confirmed triploid juveniles remained triploid. There were no confirmed triploid males. The individual whose hematopoietic cells remained triploid was a female that had only atretic follicles (absence of vitellogenic follicles). Of the individuals who lost triploid status, all males had spermatozoa, 25% of the females had a predominance of atretic follicles, and 75% had a predominance of vitellogenic follicles. In the treated group, all males had spermatozoa, 66.7% of females had a predominance of vitellogenic follicles and 33.3%, a predominance of atretic follicles. For the gonadosomatic index (GSI), males and females were analyzed separately. The only animal that was confirmed triploid was a female and her GSI was 0.19. For the remaining individuals there was no difference between treatments. Our results demonstrate that there is still much to be investigated about chromosome manipulation as a sterility tool for tilapia and other fish. Furthermore, we highlight the risks in the production of fish considered triploids, in which their ploidy condition was assessed only in the initial stages of life. Loss of triploid status can be a problem for biological control and commercial aquaculture. The elimination of a set of chromosomes during triploid somatogenesis may have the evolutionary significance of restoring genome balance close to the normal state. This loss in tissues with a high proliferation rate, such as the gonads, can result in diploid germ cells, which can lead to the formation of fertile haploid gametes. In other words, reproductive capacity can be recovered in animals originally considered sterile. It is important to reevaluate the feasibility of using triploids as an option for Nile tilapia production or even as a research model.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

A prospective study of the proteome of equine pre-implantation embryo

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The present study was conducted to investigate the global proteome of 8-day-old equine blastocysts. Follicular dynamics of eight adult mares were monitored by ultrasonography and inseminated 24 hours after the detection of a preovulatory follicle. Four expanded blastocysts were recovered, pooled, and subjected to protein extraction and mass spectrometry. Protein identification was conducted based on four database searches (PEAKS, Proteome Discoverer software, SearchGUI software, and PepExplorer). Representation of pathways was analyzed using Panther, BlastKoala, and String platforms. After the elimination of identification redundancies among search tools (at three levels, based on identifiers, peptides, and cross-database mapping), 1977 proteins were reliably identified in the samples of equine embryos. These proteins were primarily associated with cellular and metabolic processes, localized within cells, organelles, and protein-containing complexes. Our analysis identified six critical pathways related to embryo development, encompassing the TCA cycle, pyruvate metabolism, glycolysis, purine metabolism, pentose phosphate pathway, and pathways governing cell-cell communication and extracellular matrix remodeling. Additionally, pathways related to intracellular remodeling, such as cytoskeleton regulation by Rho GTPase, were observed. In conclusion, our study provides valuable insights into the complex interplay between energy metabolism, extra-cellular matrix (ECM) interactions, and embryonic development in horses, shedding light on the roles of metabolic adaptations in embryonic progression.

Keywords: equine blastocysts; embryo development; maternal-embryo interactions; proteomics.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

The effect of two vitrification protocols of ovine ovarian tissue after 14 days of *in vitro* culture

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Ovarian tissue cryopreservation is a crucial alternative for preserving the fertility of high-value animals that die unexpectedly. Vitrification has been widely explored among cryopreservation methods due to its practicality, speed, and economy, resulting in successful births after warming and autotransplantation [1]. However, despite advances, its success is still limited, and no established standard protocol exists. Therefore, careful selection of an ovarian tissue vitrification protocol and device is essential to ensure an adequate cooling rate and avoid cryoprotectant toxicity. The objective of this study was to compare two protocols for vitrification of sheep ovarian tissue, using straws and the OTC device (Ovarian Tissue Cryosystem), followed by *in vitro* culture for 7 or 14 days. We collected ovaries from five mixed-breed sheep, from which we obtained twenty-one fragments (n=21) from each pair of ovaries. These fragments were distributed into fresh groups fixed immediately (Control, n=3); cultured *in vitro* for 7 days (CIV7, n=3) or 14 days (CIV14, n=3); vitrified in straws (VS, n=6) and OTC (OTC, n=6). After warming, the vitrified fragments were cultured *in vitro* for 7 (VS7, n=3; OTC7, n=3) or 14 days (VS14, n=3; OTC14, n=3). All fragments were intended for histological analysis (follicular morphology), immunohistochemistry for cell proliferation (KI67), and measurement of Reactive Oxygen Species (ROS) levels. The data were statistically analyzed using Sigma Plot software, with comparisons between treatments made using the ANOVA test followed by the LSD test, considering significance when $P < 0.05$. The results showed that both vitrification protocols significantly reduced ($P < 0.05$) the percentage of morphologically normal follicles compared to the control group, as expected, due to cryoprotectant cytotoxicity and rapid cooling rates [2]. However, after *in vitro* culture, we observed that the vitrification protocol with OTC, followed by culture for 7 or 14 days (OTC7 and OTC14), helped maintain follicular normality, presenting follicles morphologically similar to the CIV7 group. We believe that, in addition to the OTC device, which favors cooling due to its stainless-steel material, the combinations of cryoprotectants (Ethylene glycol + Dimethyl sulfoxide) used also contributed to these results [3]. Ki67 protein immunostaining in preantral follicles revealed greater proliferative potential after 7 days of culture in both vitrification protocols compared to the control. However, there was a significant reduction ($P < 0.05$) in the VS14 group compared to VS7, suggesting a possible limitation of VS14 in supporting a long culture period. Furthermore, the evaluation of ROS levels in ovarian tissue showed that the VS14 protocol increased ($P < 0.05$) oxidative stress, which was not observed with the OTC14 protocol. We hypothesize that the presence of alpha-lipoic acid in the OTC protocol may have contributed to the reduction in excessive ROS generation. In conclusion, the use of the OTC device can be an excellent option to ensure functional recovery and follicular survival after vitrification, warming, and *in vitro* culture of ovarian tissue.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

The role of leukocyte infiltrate in cervical opening in bitches with pyometra – Preliminary Results

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Pyometra is a disease caused by bacterial colonization of the uterus, related to hormonal and structural changes during the estrous cycle in dogs. The severity of clinical signs has been associated with cervical status. More severe organic dysfunctions are related to cervical closure; therefore, the cervical opening phenomenon plays a crucial role in pyometra prognosis. Mechanisms regulating cervical collagen remodeling are very complex. Considering previous evidence that leukocyte infiltration and extracellular matrix remodeling play an important role in the physiological process of cervical opening during the estrous cycle, this study aimed to compare leukocyte infiltration in dogs with open cervix pyometra (OCP) or closed cervix pyometra (CCP). Protocol approved by the Ethics Committee - 012.2022. Fourteen pyometra cervix samples (7 OCP and 7 CCP) were selected from emergency ovariohysterectomy at an institutional hospital for standard histological processing (H&E staining) and electron microscopy. During analysis, the following histological layers were evaluated: superficial epithelium, lamina propria, and muscular layer. In the lamina propria of the OCP group, there was a significant number of dilated blood capillaries, and interstitial edema, causing separation of cells and collagen fibers, with frequent eosinophilic infiltrates. Macrophages and foreign-body giant cells were also abundantly observed. In contrast, in the CCP group, a well-defined structural pattern was observed, with aligned collagen fibers and a scarce number of leukocytes distributed across layers. Electron microscopy reinforced the finding of homogeneity in the lamina propria of CCP animals, mainly composed of fibroblasts, with occasional presence of macrophages and polymorphonuclear cells. Conversely, in the OCP group, a diversified cellular population was evident, with eosinophils, macrophages, foreign-body giant cells, and mast cells frequently found alongside fibroblasts. Notably, leukocyte characteristics in OCP included eosinophils in degranulation process and macrophages filled with vacuoles, indicative of high phagocytic activity. It is known that eosinophils' specific granules contain metalloproteinases capable of digesting collagen fibers, along with other substances with pro-inflammatory activity. Throughout most of estrous cycle, lamina propria of uterine cervix in normal dogs presents rare eosinophils. However, eosinophil count doubles during estrus, associated with vasodilation and interstitial edema, coinciding with physiological cervical opening, as observed in OCP. Therefore, it can be deduced that the stimulus for cervical opening in pyometra may occur via a non-hormonal pathway, i.e., the phenomenon may occur through activation of inflammatory pathway, potentially involved in collagen degradation and connective tissue remodeling.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Application of mesenchymal stem cells in the ovaries of prepubertal heifers challenged with *in vitro* embryo production: preliminary results

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Mesenchymal stem cells (MSCs) are cells that can be acquired from adipose tissue and cultivated. They have a high degree of plasticity and therapeutic effect as they modulate inflammation by producing a large number of bioactive molecules such as adhesion molecules, extracellular matrix proteins, cytokines and receptors for growth factors, allowing them to interact with other cells. These molecules act by modulating the inflammatory response, angiogenesis and mitosis of the cells involved in the tissue repair process and reducing the formation of fibrous tissue. *In vitro* embryo production has been the technique used on a large scale to multiply females of high genetic value. The production of embryos from heifers has proved to be a challenge in terms of producing freezable embryos. Studies have shown that the use of stem cells is safe and effective for cows calving under normal field conditions. MSCs can produce vesicles in a paracrine manner, which will allow for better performance of the oocytes produced and, consequently, of the frozen embryos. The aim of this study was to verify the effect of MSC application on the *in vitro* production of embryos (IVPE) in prepubertal heifers. Therefore, 21 heifers were selected to undergo the oocyte harvesting procedure. Immediately after harvesting, 3 million MSC were applied to the cortical region of each ovary using an ultrasound-guided needle. After 40 days, the females were subjected to a second collection of oocytes. The oocytes from both harvests were taken to the Bio Animal Reproduction laboratory for maturation and *in vitro* fertilization. Semen from the same bull and the same departure was used in both repetitions, so that there was no male effect. Comparisons between the treated group (with MSC) and the untreated group (control - without MSC) were made using the T-test and a probability of $P \leq 0.05$ was considered significant. Data are presented as mean ± standard error of the mean (SEM). The average number of oocytes, embryos and cleavage percentage recovered in the MSC-treated group was 23.14 (±4.54), 4.19 (±0.97), 15,66% (±2.52) respectively, while in the untreated group it was 16,4 (±2.82), 3.85 (±0.67), 24,85% (±5.00). No statistical difference was observed in any of the variables analyzed. In conclusion, the study is ongoing and it is necessary to test the application on a larger number of animals in order to have a significant sample size. In addition, we intend to evaluate other responses to the application of MSC, such as the influence of the treatment in preventing the formation of lesions and fibrosis in animals submitted to the oocyte harvesting procedure from the prepubertal stage onwards.

Keywords: bovine; cell therapy; reproductive efficiency.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Validation of protocols for fixed-time transcervical and laparoscopic artificial insemination in goats from the Northeastern Dry Forest of Peru

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In goat breeding within the northeastern dry tropical forest of Peru, the lack of specialized breed stallions and their high-cost act as constraints on the genetic advancement of herds. The challenge of replacing old stallions increases inbreeding, making genetic improvement programs difficult and slowing herd progress. Artificial insemination emerges as a fundamental tool to improve genetics and optimize reproductive handling. The AI facilitates the scheduling and continuous control of reproductive services throughout the year. Various synchronization protocols of different durations (1) are used, commonly involving the use of prostaglandins (2). The main objective of this research was to validate protocols for fixed-time artificial insemination, both transcervical and laparoscopic, in Creole goats from the dry tropical forest of Peru. The research was carried out in the northeast of the country, in the Amazonas region. A total of 119 goats were inseminated with frozen Boer semen, 56 by transcervical insemination, and 63 by laparoscopy. Different synchronization protocols were applied, evaluating three doses of the equine chorionic gonadotropin (eCG) hormones: 200 IU, 300 IU, and 400 IU. Two-time intervals were also tested within the insemination technique: 38 and 42 h for the transcervical technique, and 44 and 48 h for the laparoscopic technique. On day 0, a Sincrogest® intravaginal sponge was placed; on day 10 the eCG was administered intramuscularly, and on day 12 the sponge was removed. Subsequently, transcervical insemination was performed 38 or 42 h after removing the sponge, and laparoscopic insemination was performed 44 or 48 h later, prior 24-hour fasting. The diagnosis of pregnancy was carried out by transrectal ultrasonography 50 days after transcervical insemination and 49 days after laparoscopic insemination, using an Esaote® ultrasound equipment (MyLab One model). There were no notable differences ($p > 0.05$) between the two insemination techniques, the eCG dosage, and the timing of insemination. The overall pregnancy rate was consistent at 75% for both techniques. However, when examining the numerical data related to eCG dosage, it was observed that the 400 IU dose resulted in a higher pregnancy percentage (80%). Additionally, within each technique, the optimal timing for insemination differed: transcervical insemination showed better results at 38 hours (81%), while laparoscopic insemination performed best at 44 hours (77%). Combining technique, eCG dosage, and timing, the protocol that yielded the highest success rate in native goats was 200 IU at 38 hours for transcervical insemination (87.5%) and 400 IU at 44 hours for laparoscopic insemination (81.8%). In conclusion, the research demonstrated that fixed-time artificial insemination is a fundamental tool to improve genetics and optimize reproductive management in creole goats in northeastern Peru. The dose of 200 IU of eCG at 38 h for transcervical insemination and 400 IU at 44 h for laparoscopic were the most successful protocols in terms of pregnancy rates.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Uterine ozone therapy as an auxiliary diagnostic and treatment tool in mares suffering from subclinical endometritis

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Endometritis is known for being one of the major causes of infertility in the mare. Recently, intra uterine ozone therapy (IUOT)has been studied as an auxiliary treatment by reducing both inflammation and uterine infection. [1,2,3] The aim of this study was to evaluate the efficacy of intrauterine ozonized oil infusion (Sunflower oil, 600mEq/Kg) in subfertile mares showing no clinical signs of endometritis. Throughout the 2021-22 breeding season, 16 Arab mares, aged 6 to 16 years, with history of infertility in the previous two years were selected for the study. All the mares selected were evaluated by uterine cytology and low-volume lavage to discart uterine bacterial infection before IUOT infusion. Cytology and culture samples were collected during estrus, when mares showed at least one follicle bigger than 30mm and uterine edema of 2 to 3 (on a scale of 0 to 5). Half of them (n=8) presented a small amount of anechoic intrauterine fluid (IUF; less than 15mm in diameter) at the time of sampling. At the eight-day post ovulation, using a flexible artificial insemination pipette, mares were infused with a total of 26mL of sterile ozonized oil (a sample was collected for culture, certifying that there was no contamination), divided equally between each uterine horn, and injected with 250 µm of PGF2α (dinoprost tromethamine). Twenty-four hours later, mares were evaluated through rectal ultrasonography to assess the presence IUF, which was recovered through uterine lavage with saline solution (NaCl 0,9%) and submitted to culture. All samples were sent for evaluation to Sharjah Equine Hospital Laboratory, Sharjah, United Arab Emirates. The results were compared using Fisher's exact test ($P<0.05$). Positive bacterial culture rate was 62.5% (10/16), all to Streptococcus zooepidermicus (100%; 10/10), in which only samples from mares showing inflammatory IUF (81.25%; 13/16) 24 hours after IUOT resulted in bacterial growth (76.92%; 10/13). Mares that did not present IUF (n=3) were negative for bacterial culture. The 10 positive mares received antibiotic therapy and 9 (90%) became pregnant after treatment. According to Zobel and Tkalčić, [4] intrauterine treatment with ozone provides a more favorable environment for insemination and fertilization, by reducing inflammation from endometritis. The mechanism of action occurs by reducing the inflammatory condition, improving the perfusion of injured tissues, and activating the immune system based on the production of cytokines. [4] Additionally, in this study, IUOT stimulated an inflammatory reaction in the uterus of mares with subclinical infection, as ozone itself causes an inflammatory reaction in the endometrium, however, without harming it or impacting the pregnancy rates of healthy mares. [5] Our hypothesis is that ozone, by inducing a mild endometrial inflammation, promoted the shedding of the endometrium, which may have harbored Streptococcus zooepidermicus, masking its detection by conventional culture methods. According to Skive et al. [6], this bacterium has an intracellular phase in its pathogenesis, which would explain its ability to internalize and survive in endometrial cells, remaining hidden in the initial lavage performed in this study. This characteristic also explains its capacity to cause recurrent/persistent infections. Petersen et al. [7] also demonstrated that endometritis caused by Streptococcus zooepidermicus may be underdiagnosed due to the reservoir of these dormant bacteria residing in the endometrium. Therefore, we recommend for future studies the performance of endometrial biopsies before treatment with ozone-treated sunflower oil to confirm the hypothesis raised. Articles presenting results of endometritis treatment in mares using ozone therapy are still scarce, highlighting the importance of further studies with ozone formulations in equine reproduction.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Reproductive performance of small ruminants submitted to estrus synchronization with cloprostenol and reduced GnRH dose

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Manipulating the estrous cycle is a tool used to increase the reproductive efficiency of the herd. In small ruminants, the use of gonadotropin-releasing hormone (GnRH) in association with prostaglandin is used in order to improve synchronization efficiency by increasing fertilization rates. Therefore, the objective of the study was to evaluate the application of a reduced dose of GnRH in cyclic ewes and goats submitted to a hormonal protocol based on cloprostenol. Thus, the study was carried out at the Department of Animal Science at the Federal University of Ceará (Fortaleza-Ceará; -3°44'33"; -38°34'33"). For this purpose, six ewes (34.2 kg; 2.4 years; BCS: 3.5) and five does (39.6 kg; 3.4 years; BCS: 3.4) were allocated in outdoor collective pen (140 m²; shade: 16 m²), by species. Females were fed according to their body weight requirements, and mineral salt and water were offered to animals as a free choice. The hormonal protocol consists of a double application of cloprostenol (1 mL of Ciosin®, MSD; 0.25 mg of cloprostenol) on day zero (D0) and day nine (D9), with the potential application of a third dose on day 15 (D15), for those females that did not show estrus up to D15. From D10 onwards, a sexually active male was taken to the females pens to detect estrus in the morning (09:00 a.m) and afternoon (03:00 p.m), under the supervision of the handler, so the female was considered to be in estrus when it showed the immobilization reflex. On day of estrus was applied, intramuscular, 0.5 mL of GnRH (Gestran Plus, Tecnopec; 0.0125 mg of gonadorelin acetate), and female was then directed to mating, both in the shift in which estrus was verified and in the subsequent turn. After 90 days, an ultrasound was performed to check pregnancy. Thereby, the percentage of animals in estrus, duration of estrus (in hours), the pregnancy rate, birth rate, prolificacy, gestation period and weight of the offspring at birth were calculated. As a result of the hormonal protocol, 50.2 hours after the end of the protocol, 100% of the evaluated sheep showed estrus, lasting less than 24 hours. All females gave birth after an average gestation time of 148.8 days, with a prolificacy of 1.0 and the offspring weighing 2.86 kg. It is noteworthy that 3 sheep received a third dose of cloprostenol on D15 because they did not show estrus in the days following D9, showing that in some sheep reduced doses of cloprostenol started luteolysis but were unable to complete the process (1). On the other hand, all goats became estrus 81.6 hours after the second application of cloprostenol, but only 40% became pregnant with an average gestation time of 146.5 days; presenting a prolificacy of 2.0, and offspring weighing 2.4 kg. These results corroborate the observations that a half dose of gonadorelin acetate is capable of triggering an LH response sufficient to promote ovulation (2). Hence, it can be concluded that the association of a reduced dose of GnRH in association with cloprostenol, although inefficient for cyclic goats, presented beneficial effects on the reproductive efficiency of cyclic sheep raised in a semi-humid tropical climate.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Validation of uterine explants culture from female dogs in diestrus

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The frequent incidence of uterine problems in female dogs in diestrus, demands deep studies for affections comprehension and prevention. Ethical and operational questions limit animal studies, therefore, the explants' culture comes up as an alternative to reproductive system studies, since it allows experiments controlled by the researcher to be carried out in a lower space and time, with a reduced number of animals without a reduction of experimental repetitions (1,2). The explants cultivate or ex vivo method is based on the cultivation of tissue fragments obtained with a punch or scalpel and subsequently cultivated in a medium culture with a similar body temperature, resembling *in vivo* conditions. This study aimed to standardize an experimental model for female dogs by uterine cultivation *in vitro*. Ten (n = 10) uterus were obtained from healthy females aged between one and eight years old, submitted to ovariohysterectomy in the diestrus phase, confirmed by reproductive historic and vaginal cytology. After the surgery, the macroscopically healthy uterus was washed with Mili-Q water, and the uterine large ligament was excised. Subsequently, both uterine horns were assessed longitudinally after a transversal incision on each horn and submersed in a cultivated medium, where the fragments were obtained with the help of a 6mm punch. For histopathological analysis without incubation (0h), two explants were selected, and another two were incubated at 38ºC in a cultivated medium DMEM high glucose (Dulbecco's Modified Eagle Medium – powder high glucose, gibco®) enriched under agitation for 24h in a total of 40 explants. The cultivation was carried out in culture plates containing 5mL of cellular cultivate medium (DMEM) supplemented with fetal bovine serum (10%), transferrin insulin in selenium (10%), multivitamin complex (10%), and antifungal-antibiotic (1%) containing 10,000UI/mL of penicillin, 10,000µg/mL of streptomycin and 25µg/mL of amphotericin B. After incubation, the explants were processed for morphological analysis with a lesional score. The histological alterations were classified using a score varying from 0 to 3 (according to intensity or lesion frequency) times the gravity factor. The cellular homeostasis parameters were assessed in the supernatant of the cultivation medium, collecting 1mL before and after the incubation and analyzed by blood gas analysis (Siemens - RAPIDPoint® 500 Blood Gas Systems). The most frequent histological findings in both groups were interstitial edema and lining epithelium flattening. There was no difference ($P>0.05$) in the lesional score between the 0h and 24h groups. Despite of short incubation time, only soft degenerative was observed which possibly is associated with a relative hypoxia degree during the explant culture, which was commonly observed in a similar study in ovarian tissue (4). In the supernatant analysis, a difference ($P<0.05$) was observed in the osmolarity (decrease), lactate dosage (increase), and glucose levels (decrease). It was possible to constate that cultivating medium was effective mainly by the glucose consumption with consequent osmolarity decreased and lactate increased. These changes are predictable in an ex vivo context, given the relative hypoxia (1). The ex vivo uterine explant model in female dogs is suitable, contributing to the animal bioethics and the 3Rs (replacement, reduction, and refinement) conception.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

YAP-TEAD Interaction Alters Adipokine Receptors Expression in Bovine Luteal Cells Culture

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Maternal recognition of pregnancy (MRP) in ruminants is characterized by the embryo signaling to the mother through the production and secretion of Interferon Tau (IFNT) (1). Studies have already demonstrated the expression of interferon-stimulated genes (ISGs) in extrauterine tissues, including the corpus luteum (CL) (2). Several studies suggest the occurrence of an interaction between IFNT signaling and the activity of YAP (yes-associated protein), one of the effectors of the Hippo signaling pathway (3). The Hippo signaling pathway is considered a highly conserved pathway with well-defined functions in organ size determination, cell differentiation, proliferation, and apoptosis (4). Additionally, multiple pathways are involved in the development of adipose tissue, and recent studies suggest the involvement of the Hippo pathway (5). This tissue is also responsible for the production of adipokines, hormones that also play a role in certain reproductive processes (6). Therefore, our study hypothesizes that the YAP-TEAD interaction alters adipokine receptor transcription in luteal cells. The objectives of this study were to evaluate the influence of blocking the YAP-TEAD interaction on the expression of adipokine receptors in bovine luteal cell culture treated with IFNT. For this, a primary culture of bovine luteal cells was performed. Cells were dissociated and placed in 60mm plates (24 wells) in culture for 24 hours at 37ºC with DMEM-F12 presented 75% viability (Trypan blue). After this, media was replaced and cells treated according to five groups as follows: Control group (500uL DMEM-F12), group 1 (1ng/mL of roIFNT), group 2 (1ng/mL of roIFNT + 0,1 µM of VP), group 3 (1ng/mL of roIFNT + 0,5 µM of VP) and group 4 (1ng/mL of roIFNT + 1,0 µM of VP). Luteal cells were treated with VP for 1 hour before IFNT treatment for 6 hours. After culture, media and cells were collected and stored at –80ºC. Total RNA was extracted using PureLink™ RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (100ng) was reverse transcribed (RT) using the iScript™ cDNA Synthesis Kit (Bio-Rad, Des Plaines, IL, USA). It was evaluated the expression of steroidogenic enzymes (P450sCC and 3βHSD), interferon-stimulated genes (ISG15, MX1, and MX2), and a target gene of the Hippo signaling pathway (CTGF). The relative expression of P450sCC and 3βHSD presented no difference ($P > 0.05$) in all groups, these results validate the luteal culture model. The relative expression of interferon-stimulated genes was greater in group 1 when compared with the control group ($P < 0.05$), validating the pregnancy model. The CTGF levels were reduced in a dose-dependent manner among the different concentrations of VP, confirming the inhibitory effect of VP on the YAP-TEAD interaction. The expression of GRP78 was greater ($p < 0.05$) in the group treated with 1 µM VP compared to the remaining groups. Expression of ITGB1 levels was reduced in a dose-dependent manner ($P<0.05$) among the different concentrations of VP. Our results present that blocking the YAP-TAD interaction modulates the gene expression of the adipokine receptors ITGB1 and GRP78 in bovine luteal cells during the early gestation period.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Efficacy of the Ovsynch protocol in cyclical goats raised in northeastern Brazil

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The use of fixed-time insemination precedes the application of estrus and ovulation synchronization protocols. Thus, the Ovsynch protocol is an alternative for use in goats given its ease and reduced cost compared to intravaginal device protocols. Therefore, this study aimed to evaluate the efficacy of the Ovsynch protocol on the occurrence of estrus in cycling does raised in northeastern Brazil. This study was performed at the Animal Science Department of the Federal University of Ceara, Fortaleza-Ce (-3°44'33"; -38°34'33"). Therefore, were used 11 fertile cycling goats (35,2 kg; 5 years), previously evaluated by ultrasound, allocated in a collective pen of 140 m², and fed with Tifton grass hay, concentrated feed, and mineral salt and water ad libitum (NRC, 2007). The Ovsynch treated does received an intramuscular injection of a GnRH analogue (0,004 mg buserelin; Sincroforte®, Ourofino), on day 0. On day 7, goats were treated with an injection of PGF2α analogue (0,5 mg d-cloprostenol; Estron®, Agener União), followed by a second injection of 0,004 mg buserelin, after a 48 hours period. After 30 hours the last GnRH analogue injection was performed transcervical artificial insemination using refrigerated goat semen in all experimental goats. To assess the onset and duration of estrus goats were teased with a sexually active buck accompanied by a handler, at 9:00 a.m and 3:00 p.m, from D10, so the goat was considered to be in estrus when it showed the immobilization reflex (the classical indicator of estrus). The Ovsynch protocol resulted in the occurrence of estrus in only 36.3% of does after 24 hours of the last hormone injection, so 72% of the females showed estrus until D13, with 100% of estrus being accumulated only at D18. The first estrus duration was 50.2 ± 8.84 hours (min: 12 hours; max: 96 hours). Furthermore, 36% of the goats showed repetition of estrus, which occurred after 9.3 ± 1.76 days (min: 3 days; max: 16 days) and was characterized as a short cycle. Although all goats showed estrus as a result of the Ovsynch protocol, the synchronization degree was below expectations. Using the Ovsynch protocol, Holtz et al. (1) related 96% of estrus, starting 49.3 hours after the end of the protocol, with an estrus duration of 35.7 hours, verifying the occurrence of corpus luteum with premature regression (29%). Also, Riaz et al. (2), when subjecting goats to the Ovsynch protocol, reported a total of 71% of females in estrus, with an interval of 48.0 hours between the application of PGF and the occurrence of estrus and 73.0 hours before the occurrence of ovulation, with estrus duration of 44.7 hours. Therefore, it is concluded that the Ovsynch protocol does not present itself as a viable option for use in fixed-time artificial insemination of cyclic goats raised in northeastern Brazil, given its low effectiveness in synchronizing the occurrence of estrus, requiring further studies to adjust doses and frequency to use in fixed-time AI (artificial insemination) protocols for goats effectively.

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X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Characterization of estrus and monitoring of follicular dynamics of Brazilian Northeastern Ecotype Donkeys

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In Brazil, the semiarid northeastern region covers an area of 982,563.3 km² and comprises 1,133 cities, with approximately 22 million population. This region is characterized by the presence of the Caatinga biome, a predominant ecosystem in the region, occupying 70% of the Northeast region of Brazil. In Brazil, the Northeast region concentrates 80% of the population of approximately 862,000 donkeys (FAO, 2019). In addition to the inherent adaptability to this region, donkeys were subjected to a process of natural selection and developed specific adaptation characteristics. This is how the Brazilian northeastern ecotype donkey emerged. The mechanization of agriculture, associated with the migration of people from the countryside to towns have led to a reduction in the number of donkeys and their importance. In recent decades, the Brazilian donkey population has followed the same global trend of reduction. Due to the decrease in interest in the use of the donkey species in agricultural practices, many animals were abandoned, living in almost wild conditions. In this sense, a new productive/economic activity for the species may be necessary for preserving the northeastern ecotype donkey. Donkey milk production is already a reality in several parts of the world and could be an attractive alternative. Given the imminent development of the species' production chain in the country, studies on the reproductive characteristics of this ecotype are necessary. Estrus is the period of the estrous cycle characterized by the female's acceptance of the male's mount. Equine females have an estrus phase lasting 5 to 9 days, requiring monitoring of follicular dynamics to determine the moment of ovulation. In this sense, the present work aimed to evaluate the main behavioral characteristics of female donkeys from the northeastern ecotype, as well as to monitor follicular dynamics during estrus. Seventeen Brazilian Northeastern Ecotype Donkeys were clinically and gynecologically previously selected. To determine behavioral parameters, females were teased daily in the morning. According to their behavior during teasing, they were classified into three categories 1 (absence of estrus); 2 (weak estrus) and 3 (intense estrus). Evaluating only females in estrus, phases 2 and 3, the following behaviors were observed: in phase 2, females were passive towards the male, but without opening their pelvic limbs and with a discreet presence of the movement of opening and closing their mouth, Commonly called "chewing". In phase 3, the females presented vocalization, kept their ears down close to the neck, opened their pelvic limbs, chewed intensely, and accepted being mounted. In parallel, the animals were monitored daily with B-mode transrectal ultrasonography, to evaluate the ovarian follicles, which were measured daily and noted on a control spreadsheet. For follicular growth, descriptive statistical analysis was used, and it was observed that on average, donkeys of the northeastern ecotype showed a mean follicular growth of 2.5 mm per day, during estrus, and that this phase of the estrous cycle presented an average of 6 to 7 days. It is concluded that the estrus of donkeys of the northeastern ecotype is similar, in duration, to that of other equid females, with daily follicular growth of 2.5 mm and important behavioral signs for identifying estrus.

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FEMALE REPRODUCTIVE BIOLOGY

Evaluation of different dosages of dinoprost tromethamine as luteinizing agent in Brazilian Northeastern Ecotype Donkeys

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In Brazil, the semiarid northeastern region covers an area of 982,563.3 km² and comprises 1,133 cities, with approximately 22 million population. This region is characterized by the presence of the Caatinga biome, a predominant ecosystem in the region, occupying 70% of the Northeast region of Brazil. The Northeast region of Brazil concentrates 80% of the population of approximately 862,000 donkeys (FAO, 2019). In addition to the inherent adaptability to this region, donkeys were subjected to a process of natural selection and developed specific adaptation characteristics through random mating. This is how the Brazilian northeastern ecotype donkey emerged. The mechanization of agriculture, associated with the development of the automobile industry and migration of people from the countryside to towns have led to a reduction in the number of donkeys and their importance. In recent decades, the Brazilian donkey population has followed the same global trend of reduction. Due to the decrease in interest in the use of the donkey species in agricultural practices, many animals were abandoned, living in almost wild conditions, becoming one of the main causes of automobile accidents on the roads of the Northeast region of Brazil. In this sense, a new productive/economic activity for the species may be the only alternative for preserving the northeastern ecotype donkey and reducing road accidents. Luteolysis is the process of destruction of the corpus luteum (CL), a temporary structure formed in the ovary after ovulation, resulting in the interruption of progesterone production. This occurs to prepare the female reproductive system for a new estrous cycle. The equine CL has an 18-fold greater sensitivity as compared with the bovine. And the affinity of equine luteal cell membrane preparations for PGF2α is approximately 10 times greater than that for bovine luteal cell membrane preparations. However, there is no studies on Brazilian Northeastern Ecotype Donkeys in use of luteinizing agents. Therefore, this study seeks to analyze the luteolytic response to Dinoprost Tromethamine (Lutalyse®) at different doses in northeastern ecotype donkey. Seventeen donkeys were selected, aged between 2 and 7 years old, and were monitored daily using B-mode transrectal ultrasound. After the reproductive evaluation, donkeys were divided into two groups, the first with 8 and the second with 9 jennies. Four days after ovulation, donkeys received application of the prostaglandin Dinoprost Tromethamine intramuscularly. The first group, with 8 donkeys, received 5 mg (1mL) of Lutalyse®, while the second group, with 9 donkeys, received 10 mg (2mL). Four days after application, they underwent ultrasound evaluation, and it was found that 8 of the 9 females that received the 10 mg dose had CL lysis within the expected period of four days, different from the group that received 5 mg, which only 1 donkey presented CL lysis. These results indicate the effectiveness of Dinoprost Tromethamine (Lutalyse®) with a dose of 10 mg, in donkeys of the northeastern ecotype for inducing CL lysis. Therefore, these findings contribute to the knowledge of the characteristics and peculiarities of northeastern donkeys, allowing the implementation of preservation measures and adequate management of this species that is so important for the Brazilian semi-arid region.

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X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Xenotransplantation as an alternative for tissue revitalization for *post-mortem* somatic cell culture in jaguars (*Panthera onca*)

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The escalation of human-wildlife conflicts, as dirt road paving, has led to a significant increase in animal roadkill incidents, severely impacting endangered species such as the jaguar. In this context, genetic recovery of roadkill specimens emerges as a crucial strategy for preserving the genetic diversity of these species, aligning with the One Conservation [1] concept. Auricular cartilage tissues from three jaguars (two males – Atrop1 and Atrop2 – and one female – Atrop3), victims of vehicular collisions on BR-262 in Southern Pantanal, Mato Grosso do Sul (MS), were collected, and sent to the Laboratory of Genetic Editing and Conservation at Federal University of Mato Grosso do Sul (Reprogen/UFMS) by the Environmental Police of MS, approximately 24 hours post-mortem. The tissue processing procedures, including cryopreservation, rewarming, and histological analysis, were meticulously conducted as previously described [2]. Tissue fragments underwent xenotransplantation into immunodeficient NSG mice and were divided into groups: cryopreserved (CRIO), cryopreserved followed by intraperitoneal xenotransplantation (XenoIP), and cryopreserved followed by subcutaneous xenotransplantation (XenoSC). Quantitative histological analysis of dermal fibroblasts and subsequent *in vitro* cultivation indicated that XenoSC surpasses XenoIP and CRIO in fibroblast proliferation and preservation of normal chondrocytes. Data showed that for Atrop1, XenoSC significantly increased the number of fibroblasts and normal chondrocytes (28.4 ± 9.48 and 15 ± 5.07 respectively; $p<0.05$) compared to XenoIP (11.1 ± 5.47 and 15.8 ± 5.53) and CRIO (20.6 ± 7.17 and 7.5 ± 3.31). For Atrop2, XenoSC results (17.2 ± 7.98) were similar to fresh tissue (25.1 ± 12.6), demonstrating equivalent efficacy in maintaining cellular viability. In Atrop3, XenoSC maintained superiority in the quantity of fibroblasts and normal chondrocytes (7.4 ± 4.67 and 21.0 ± 8.08; $p<0.05$) compared to XenoIP (1.75 ± 1.86 and 15.2 ± 9.42). Furthermore, cellular viability assessments showed no significant statistical differences between XenoIP and XenoSC treatments compared to fresh tissue, suggesting both xenotransplantation approaches are effective, with a slight advantage for XenoSC in preserving cellular and tissue structures. This study demonstrates the superiority of XenoSC in revitalizing cartilaginous tissues of roadkilled jaguars, outperforming XenoIP and CRIO techniques in terms of cellular viability and fibroblast proliferation, while preserving normal chondrocytes. The cells obtained from the xenotransplanted tissues (XenoIP and XenoSC) underwent a genotyping process using the real-time PCR (Polymerase Chain Reaction) technique, employing specific primers for mice and jaguar. The DNA analysis of these samples confirmed that the somatic cells belonged exclusively to the jaguar. The technique enabled the recovery of somatic cells from jaguars in early stages of decomposition, highlighting that xenotransplantation of vitrified tissues can significantly improve tissue quality, facilitating cell culture and elevating cell quality to levels comparable to fresh tissues. The ability to vitrify tissues from roadkill animals facilitates genetic rescue in various regions of the country, allowing for the subsequent sending of these samples for cultivation and conservation of fibroblasts in specialized laboratories. These advancements open new perspectives for species conservation and conservation biology research, expanding the possibilities for enriching biobanks with high-quality genetic material for future applications in assisted reproduction techniques, such as somatic cell nuclear transfer.

Keywords: Genetic Recovery, Conservation, Roadkill, Cellular Viability, Biobank, Assisted Reproduction.

Ethics and Approvals: CEUA UFMS #1278/2023, SISBIO #75762-1, SISGEN #A1CEAD0.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

Assessing the impact of superovulation on reproductive outcomes in NOD mice: Implications for laboratory animal science and assisted reproduction

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The pursuit of innovative experimental models is driven by the need for more accurate and consistent outcomes in preclinical trials. This reflects a commitment to the 3Rs principles in Laboratory Animal Science: Reduction of the number of animals used, Refinement of methods to minimize stress and discomfort, and Replacement of animal models with validated alternatives. These principles are crucial for enhancing research practices, ensuring ethics and animal welfare. A notable example is the use of the Nonobese Diabetic (NOD) mouse in research on type 1 diabetes mellitus due to its similarity to the human autoimmune process and versatility for studies across various fields. This study aimed to evaluate the reproductive capacity of the NOD strain by comparing oocyte and embryo production between superovulated females and those not hormonally stimulated. A group of 20 pairs was observed over four reproductive cycles under monogamous conditions to calculate the reproductive index. Estrous cycle synchronization was achieved through the Whitten effect, and superovulation was induced in 20 females with an intraperitoneal administration of 7.5 IU of PMSG on day 0 and, 48 hours later, 7.5 IU of hCG, followed by mating with vasectomized males (n=10) to obtain oocytes or with fertile males (n=10) for embryo collection. A control group of females (n=40) did not receive hormonal stimulation. A progressive decrease in litter size was observed, with averages of 7.50±0.68; 5.25±0.95; 2.85±0.88, and 2.20±0.73 from the first to the fourth birth, respectively. Hormonally unstimulated females produced an average of 6.25±1.15 oocytes and 2.90±0.86 2-cell embryos on D2, while superovulated females produced 79.20±8.43 oocytes and 19.90±7.90 embryos on D2. The study assessed embryo viability in natural mating and superovulation groups. The group subjected to natural mating showed a viability rate of 20.74% ± 6.27 of the total embryos, whereas the superovulated group recorded 24.71% ± 6.26. It was found that the NOD strain, under an intensive monogamous mating regime, experienced a decrease in viability after the third birth. Although superovulation increased the number of oocytes and embryos produced, comparative analysis revealed no significant statistical differences in the percentage of viable embryos between the two groups. Therefore, superovulation proves effective in generating a higher number of oocytes and embryos with fewer animals, serving as a valuable strategy for assisted reproduction and the creation of germplasm banks for the NOD strain.

Keywords: oocyte, embryo viability, 3Rs principles, assisted reproduction

Ethics and Approvals: CEUA UFMS # 1.176/2021.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

FEMALE REPRODUCTIVE BIOLOGY

DYNAMIC CHANGES OF FOLLICULAR FLUID CELL POPULATION DURING FOLLICULAR DEVELOPMENT IN COWS

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The *in vivo* cell population changes in the ovarian follicular antrum are poorly understood in cows. Our objective was to characterize the cell populations in ovarian follicular fluid (FF) during key phases of follicular development in Holstein cows using RNA-seq technology. We used 18 multiparous, nonlactating Holstein cows aged 3-7 years, with a body condition score of approximately 3.5. Ovarian follicular development was synchronized, and post-synchronization ovulation was monitored using ultrasonography every 12 hours (MyLab30 equipped with a 7.5 MHz linear-array transducer, Esaote, Genova, Italy) every 12 hours. Follicular fluid was collected at the following phase: Pre-deviation: when the diameter of the largest follicle of the wave reached 7 mm (n=3); Deviation, when the diameter of the largest follicle reached 8.5 mm. At this stage, both F1 (n=3) and F2 (n=3) were aspirated; post-deviation: when the diameter of the largest follicle reached 12 mm (n=3); and preovulatory: when the largest follicle reached 12 mm, the cow received GnRH, and 24 h later, the FF was aspirated (n=3). After collection, follicular fluid samples were centrifuged at 2,000 x g for 10 minutes at 4°C to separate the supernatant and cell pellet, which were then stored at -80°C. RNA sequencing was performed using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA), following the manufacturer's instructions. The reference genome (Bos taurus ARS-UCD1.2) was used for alignment, and the STAR software was employed for read processing. After normalization, the DESeq2 program was used for differential expression analysis with the Benjamini-Hochberg correction for multiple testing. Genes with a log2 fold change greater than 1.5, and a false discovery rate (FDR) less than 0.05 were considered differentially expressed. The data revealed significant changes in gene expression at various stages of follicular development. Gene ontology (GO) and gene set enrichment analysis (GSEA) were used to further understand the biological significance of differentially expressed genes (DEGs). Cellular heterogeneity was analyzed using CIBERSORT, with a signature matrix derived from the murine cyclic ovary atlas. The results indicated that during the early stages of follicular development, granulosa cells (GCs) were the predominant cell type ($p<0.05$). However, as the follicle developed and reached ovulatory capacity (approximately 12 mm), the presence of myeloid cells increased. After the LH peak, there was a significant increase in immune system cells ($p>0.05$), including dendritic cells, macrophages, and T cells. The presence of these cells in the follicular fluid suggested a strong chemotactic stimulus, indicating that immune cell migration occurred despite an intact basal lamina. In non-selected follicles, after deviation, immune cells also had a massive invasion of the follicular environment as atresia progressed. In conclusion, this study provides insights into the bovine follicular fluid population's progressive changes, demonstrating a shift from predominantly GCs to immune cells as the follicle develops. Further studies are needed to investigate the mechanisms driving immune cell migration in the follicular environment and its implications for follicular development and atresia.