Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Towards functional boar spermatozoa in preparation for artificial insemination: Retrospective report sourcing boar studs submitting samples to two andrology reference laboratories in North America

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The application of artificial insemination (AI) in swine continues to become more efficient as a management tool and therefore all aspects of the practice become more critical for optimal production. One aspect of the process is the functionality of sperm as packaged in semen doses. Over time, towards higher efficiency, fewer sperm are packaged per semen dose and therefore maintaining optimal sperm function is paramount to achieve optimal fertility. Leveraging third party Andrology Reference laboratories is key to establishing a benchmark for a particular boar stud relative to producing a high quality semen product for AI that facilitates satisfactory fertility at the commercial level. This retrospective report serves to provide various sperm parameters based on several commercial boar studs that submit semen doses to laboratories for quality assessment. Semen samples (n=2136), sourcing from seven boar studs located in the mid-USA region, were evaluated at two reference laboratories (Lab A and Lab B) also located in the mid-USA region. Both laboratories reported mean percent total motility as determined by computer-assisted sperm analysis (IVOS). Both laboratories performed a full differential sperm morphology evaluation using phase-contrast microscopy (1000x magnification, oil immersion) in conjunction with a wet-mount under coverslip. Total motility results for three studs submitting to Lab A were: 87.4 (+5.7); 89.0 (+4.0); and 86.1 (+4.9) while the total motility results for four studs submitting to Lab B were: 86.9 (+4.1); 87.8 (+3.7); 87.8 (+3.6); and 87.2 (+4.0). Percent normal sperm morphology for the three studs submitting to Lab A were: 85.8 (+5.9); 85.0 (+6.3); and 82.2 (+7.5) while the Lab B results for the four studs was 82.2 (+4.9); 79.0 (+5.3); 81.0 (+4.6); and 80.5 (+5.1). As the swine industry continues to gradually reduce the total number of sperm in an AI dose, emphasis will continue to be placed on semen quality evaluation to ensure functional sperm available for fertilization.

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MALE REPRODUCTIVE BIOLOGY

Semen collection with TUMASG affects less rams' welfare than electroejaculation but does not achieve the quality of the samples collected with artificial vagina

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When the artificial vagina (AV) cannot be used for semen collection, electroejaculation (EE) is widely used as the alternative technique. However, EE is stressful and painful. Therefore, it is essential to develop alternative techniques to reduce these negative effects without affecting semen quality. In this sense, the transrectal ultrasound-guided massage of the accessory sex glands (TUMASG) requires few or even no electrical pulses to achieve ejaculation. Therefore, the aims of this study were to determine if TUMASG is less invasive than EE in rams, without affecting the semen quality, and if the semen quality of samples collected using TUMASG is comparable to those collected using AV. Semen was collected from 11 adult Corriedale rams using an AV, by TUMASG and by EE (an ejaculate/technique/animal in a Latin-square design) during the transition to the non-breeding season. During semen collection the time required for ejaculation, the total number of electrical pulses applied and the number of vocalizations were recorded. Semen samples were immediately evaluated after collection, volume ejaculated and sperm concentration were determined, and the total number of sperm ejaculated was calculated. Sperm mass motility was determined subjectively (0 to 5 scale) and percentage of motile, progressive motile and static sperm and the kinematic sperm variables (VCL: curvilinear velocity, VAP: average path velocity, VSL: straight line velocity, STR: straightness (VSL/VAP), LIN: linearity (VSL/VCL), WOB: wobble (VAP/VCL)) were assessed using a CASA system (ISAS, Proisier, Valencia, Spain). Semen collection using TUMASG took longer than with EE (6.1 ± 0.7 min vs 2.3 ± 0.8 min, P=0.005), but required fewer electrical pulses (5.4 ± 1.9 vs 15.1 ± 1.9, P=0.001) of lower voltage (maximum voltage used with TUMASG: 7 V; with EE: 10 V). Additionally, there were no differences in the number of vocalizations emitted during the different collection methods. Most of the semen variables evaluated were not different between techniques. However, the volume tended to differ (1.0 ± 0.2 mL, 1.3 ± 1.8 mL and 1.7 ± 1.9 mL, TUMASG, VA and EE, respectively, P=0.06), and sperm concentration differed according to the techniques (P=0.02): it was lower in semen samples collected by TUMASG (3727 ± 1024 × 106 sperm/mL) than in those collected by AV (7468 ± 900 × 106 sperm/mL, P=0.005) and tended to be lower than in samples collected by EE (5904 ± 944 × 106 sperm/mL, P=0.09). Sperm mass motility and the total number of sperm ejaculated also differed according to the technique (P=0.002 and P=0.02 respectively). Sperm mass motility was lower in semen samples collected by TUMASG (1.8 ± 0.5) than in samples collected by AV (4.5 ± 0.5, P=0.0005), with no difference with those collected by EE (2.9 ± 0.5). Total number of sperm ejaculated was lower in semen samples collected by TUMASG (2872 ± 2043 × 106 sperm) than by AV (9994 ± 1828 × 106 sperm, P=0.02) and EE (10156 ± 1926 × 106 sperm, P=0.02). It seems that TUMASG can be used as an alternative to EE for semen collection as it reduced the invasive handlings, not affecting the semen quality. However, the ejaculate collected by TUMASG does not reach the characteristics of that collected with AV.

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MALE REPRODUCTIVE BIOLOGY

"Air flushing" is effective for epididymal ram sperm collection

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To recover sperm from the epididymal tail, retrograde flushing method or slicing technique are usually adopted, both executed using semen extenders. It is known that ram epidydimal sperm presents differences in membrane characteristics (Gautier and Aurich, 2022;) and osmotic tolerance (Tsikis et al., 2018) compared with ejaculated sperm. Therefore, if the goal is to test different extenders using the same epididymal sample, further processing is necessary to isolate sperm from flushing media (e.g., centrifugation), leading to mechanical stress and potential sperm membrane modifications because of interaction with the medium. This study reports the effectiveness of performing retrograde flushing using air instead of medium, to recover sperm from the ram epididymal tail. Testicles with their scrotum (scrotal circumference > 28 cm) were obtained from a total of 32 slaughtered rams and transported to the laboratory into an isothermal box at environmental temperature (~ 23 °C), up to six hours after slaughter. After epididymal dissection and cleaning with saline solution, a 23 G needle coupled with a 10 ml syringe, filled with air, was inserted into the deferent duct lumen. The bottom of the epididymal tail was sectioned three to five times with a scalpel, and the epididymal tail content from four rams was pooled into a conic graduated tube. The pooled samples were transferred to a water bath (37 °C) for one minute, followed by the assessment of subjective motility (0-100%, 100× magnification under bright field microscopy), vigor (1-5 score), morphology and membrane intactness (eosin-nigrosin staining, 1000× magnification), and functional membrane (hypoosmotic swelling test, HOST). Epididymal content was obtained from 60 of 64 epididymal tails (93.8%), with the volume per pool ranging from 2 to 4 mL. Sperm trait data are presented as mean ± SEM. Sperm motility, vigor, percentage of sperm with normal morphology, intact membrane, and functional membrane were 77.5 ± 1.0% (70 to 80%), 3.5 ± 0.1 (3 to 4), 83.4 ± 1.4% (72 to 96%), 83.9 ± 1.4% (70 to 94%), and 71.9 ± 1.5% (60 to 90%), respectively. In conclusion, using air for the retrograde flushing method permits the recovery of good-quality ram epididymal sperm, which can be useful for certain experimental designs.

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MALE REPRODUCTIVE BIOLOGY

Influence of swim-up on the DNA damage rates in red-rumped agouti (*Dasyprocta leporina* Linnaeus, 1758) sperm

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The red-rumped agouti is a medium size wild rodent included in the Cavioidea superfamily. Despite these animals have an important role in their ecosystems as seed scatterers, their populations are decreasing considerably due to hunting and habitat fragmentation (1). Consequently, a collective effort has been made with the purpose of studying their germplasm and apply it in reproductive biotechniques (2). However, the manipulation of these samples can directly influence their viability, as a previous study has observed that rodent spermatozoa are very susceptible to mechanical stresses (3). Therefore, we aimed to evaluate the effect of swim-up as a classical in vitro sperm selection method on the DNA damage rates in epididymal spermatozoa of red-rumped agoutis. All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 20/2021) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 76655-1). Six red-rumped agoutis were anesthetized (50 mg/kg sodium thiopental) and euthanized (1 mL/kg potassium chloride), the testes-epididymis complexes were recovered, the cauda region was excised and the epididymal spermatozoa were obtained by retrograde flushing. The samples were divided into two groups: without selection (WS), and swim-up (SP). For SP, spermatozoa were added at the bottom of a tilted tube (45°) for 15 min and then centrifuged 300×g for 10 min. Thus, for the DNA damage evaluation, spermatozoa samples were smeared and left to dry in the air. The slides were fixed in Carnoy's solution for 3 h, incubated for 25 min in a buffer solution composed of 15 mM Na2HPO4 and 80 mM citric acid (pH 2.5) at 75 °C. Smears were stained using 0.2 mg/mL acridine orange for 10 s. A total of 100 cells per group were evaluated using fluorescence microscopy (40×). According to the sperm head color, they were classified as: intact, low, medium, or high DNA damage. (4). All data were expressed as mean ± standard error (one male/one repetition) and analyzed by ANOVA followed by Tukey test ($P < 0.05$). The percentage of intact DNA spermatozoa was maintained SP (68.8% ± 0.9) similar to the WS samples (86.2% ± 7.2, $P > 0.05$). Similar results ($P > 0.05$) were observed for low DNA damage [WS (9.0% ± 5.7), SP (8.4% ± 3.0)] and high DNA damage [WS (2.5% ± 1.1), SP (10.5% ± 5.7)]. However, we were able to observe a significant increase in the percentage of sperm with medium DNA damage in the SP group (12.1% ± 5.1), when compared to WS (2.1% ± 0.9, $P < 0.05$). These results may be correlated to the swim-up protocols, where the combination of centrifugation and longer periods in the incubator with non-viable sperm could lead to an increase in reactive oxygen species levels, thus, promoting the denaturation of the DNA strand and increasing the risk of cell apoptosis (5). In conclusion, swim-up increased the DNA damage rate in red-rumped agouti epididymal spermatozoa. These results represent an important step towards understanding the male germplasm of these animals, and how they will behave during future applications of in vitro reproduction techniques.

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MALE REPRODUCTIVE BIOLOGY

Effects of centrifugation on oxidative stress status and membrane functionality of red-rumped agouti (*Dasyprocta leporina* Linnaeus, 1758) epididymal sperm

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The epididymal sperm collection procedure includes various physical manipulations such as mixing, pipetting and centrifugations (1). These preparation protocols are crucial to eliminate contaminants such as epithelial and red blood cells, bacteria, and cell debris, which can affect the spermatozoa functionality by increasing the oxidative stress status. This stress condition has been observed in laboratory rodents (2), thus becoming a concern during the sperm cell manipulation for reproductive biotechniques in wild rodents. Therefore, our aim was to analyze the effects of centrifugation on oxidative stress status and membrane functionality of red-rumped agouti spermatozoa. All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 20/2021) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 76655-1). Six males were anesthetized (15 mg/kg ketamine hydrochloride and 1 mg/kg xylazine hydrochloride) and euthanized (1 mL/kg potassium chloride). Subsequently, sperm recovery was performed from the epididymal tail by retrograde flushing. The samples were divided into two groups: fresh sperm (FS) and centrifuged (CT). The centrifugation protocol consisted of 300×g for 3 min twice, the supernatant was discarded, and the pellet was resuspended for evaluation. To determine oxidative stress, reactive oxygen species (ROS) and intracellular glutathione (GSH) levels were quantified using 10 µM 2',7'dichlorodihydrofluorescein diacetate (H2DCFDA) and 10 µM 7-amino-4-chloromethylcoumarin (CellTracker Blue), respectively. Spermatozoa were incubated in the dark with the different probes for 30 min at 37 °C in a dry bath. The cells were analyzed by fluorescence microscopy (40×), and the arbitrary fluorescence units (AFU) were quantified using ImageJ software. For the membrane functionality evaluation, a hypo-osmotic swelling test (HOST) was conducted with a solution composed of distilled water with sodium citrate and fructose solution (50 mOsm/L). Spermatozoa were evaluated in a phase-contrast light microscope (400×, 200 cells/group). Sperms that had a swollen coiled tail were classified with a functional membrane. All data were expressed as mean ± standard error (one male/one repetition) and analyzed by ANOVA followed by Tukey test ($P < 0.05$). Centrifugation (85.9% ± 3.25) was able to preserve the functionality of the sperm membrane, with values similar to FS (90.7% ± 1.69, $P > 0.05$). Moreover, sperm from the CT group demonstrated a lower production of ROS (0.57 AFU ± 0.15) when compared with FS (1.00 AFU ± 0.42, $P < 0.05$). A GSH increase was observed on CT group (1.82 AFU ± 0.94) when compared with FS (1.00 AFU ± 0.42, $P < 0.05$). These results demonstrate that centrifugation did not cause a difference in the membrane functionality of these cells, while was capable of select better quality cells, thus increasing the levels of GSH, an important intracellular antioxidant, and decreasing the levels ROS, parameters crucial for maintaining the stability and viability of spermatozoa for subsequent reproductive techniques, such as in vitro fertilization (3). In conclusion, the centrifugation method proved effective in processing red-rumped agouti spermatozoa without causing osmotic instability and preventing the imbalance of oxidative status. These findings represent a notable stride forward of assisted reproduction biotechnologies, offering a more nuanced understanding of the appropriate handling of wild rodent's sperm cells.

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MALE REPRODUCTIVE BIOLOGY

Isolation and extended *in vitro* culture of fibroblasts from northern tiger cat (*Leopardus tigrinus* Schreber, 1775) as a step towards for somatic cell nuclear transfer

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The establishment of in vitro culture of cells derived from skin fragments has been proposed as a useful technique for the conservation of endangered species. Among the species vulnerable to extinction, the northern tiger cat requires strategies for the conservation of its genetic diversity. An interesting conservation strategy would be one aimed at the development of cryobanks for use in assisted reproduction techniques. In general, the characterization of the cells during extended culture represents an important step in the formation of these banks. Therefore, we described the extended in vitro culture of fibroblasts derived from northern tiger cat skin. All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 20/2023) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 88201-1). Then, skin biopsy (1.0 cm2) derived from ear region was recovered from one male of Sargento Prata Municipal Zoo (Fortaleza, Brazil), after anesthetized. In the laboratory, fragments (6.0 mm³) were cultured under controlled atmosphere (38.5°C, 6.5% CO2) in minimal essential medium modified by Dulbecco (DMEM) supplemented with 10% fetal bovine serum and 2% antibiotic-antimycotic solution and monitored every 24 h. Cells isolated from tissues were evaluated after the third, seventh and eleventh passage for viability with trypan blue assay, metabolic activity using 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide, proliferative activity using population double time (PDT), apoptotic levels with acridine orange and ethidium bromide, reactive oxygen species (ROS) levels and mitochondrial membrane potential (ΔΨm) using H2DCFDA and MitoTracker Red probes, respectively. Additionally, cells in the third passage were evaluated for identification of your karyotype and confirmation of cell type using primary anti-vimetin antibody. All data were presented descriptively. Thus, the total culture time was 20 days for the samples derived from a male. The initial four fragments submitted to the culture all presented adherence on the first day with cell growth around the explants from the eighth day. Cells reached 70% confluence forming monolayer on day 17. During the third passage, the cells were analyzed with primary anti-vimetin antibody, identifying them as fibroblasts based on the presence of intermediate filaments. Moreover, karyotype evaluation was in conformance with previous reports on this species, with 2n = 36 chromosomes, 34 of which were autosomal, plus 2 sex chromosomes. Regarding to viability using trypan blue, values above 80% were observed in all passages (third: 80%, seventh: 97%, and eleventh: 82%). However, a numerical reduction was observed in metabolic activity during passages (third: 79.3%, seventh: 79.3%, and eleventh: 27.4%). Moreover, PDT values were similar between the third (31.7 h) and seventh passages (29.0 h), while due to the low cell concentration presented in the eleventh passage, this parameter was not quantified. Regarding to apoptosis levels, values above 95% were observed in all passages for viable cells (third: 99%, seventh: 99%, and eleventh: 98%). Additionally, ROS levels, evaluated in arbitrary fluorescence units (AFU), were maintained in 1.00 AFU during all the passages. Values of ΔΨm varied between passages (third: 1.23 AFU, seventh: 2.81 AFU, and eleventh: 2.68 AFU. In summary, we were able to isolate somatic cells derived from northern tiger cat skin, associated with high viability rates after extended in vitro culture. These results described, at first, the cellular characteristics according to different quality parameters of Northern tiger cat samples during extended in vitro culture, providing a valuable source for assisted reproduction techniques, aiming at the genetic conservation of this species.

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MALE REPRODUCTIVE BIOLOGY

Paternal treatment with kisspeptin-10 improves offspring reproductive and metabolic changes caused by hypothyroidism in male rats

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The intergenerational influence of paternal hypothyroidism is still poorly understood. However, studies have already demonstrated that hypothyroidism in males compromises gonadal morphology, sperm quality, and testicular expression of kisspeptin, a key peptide for reproduction. The objective of this study was to evaluate whether hypothyroidism in male rats compromises the sexual development and glycemic metabolism of offspring and whether treatment with kisspeptin-10 (Kp10) could prevent these effects. To establish the F0 generation, adult male Wistar rats euthyroid (n = 12), hypothyroid (n = 11) or hypothyroid treated with Kp10 (n = 12) were maintained for three weeks with healthy female rats. Hypothyroidism was induced for three months by the oral administration of propylthiouracil (PTU; 4 mg/Kg/day), with the administration of Kp10 (12 µg/Kg/day) concomitant with the last month of treatment with PTU. At the end of the mating period, all males were euthanized and had no contact with their mothers or offspring. The body development of the F1 offspring was evaluated for two months. For females, vaginal opening and cyclicity were monitored, and uterine and ovarian morphology and plasma profiles of luteinizing hormones (LH) and progesterone (P4) were evaluated at 60 postnatal days (PND). In males, in addition to the body growth and morphology of the genital tract, the plasma LH and testosterone profile was evaluated at 60 PND. To evaluate glycemic metabolism, glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed at 30 and 60 PND in males and females, and the pancreas and liver were collected at 60 PND. Regarding body mass gain, paternal hypothyroidism did not affect female growth until sexual maturity. However, it delayed vaginal opening, increased uterine weight and myometrial thickness, and reduced ovarian weight ($P<0.05$). Furthermore, it increased insulin resistance at 30 PND, caused glucose intolerance at 60 PND, and increased pancreatic mass ($P<0.05$). In males, on the other hand, paternal hypothyroidism increased body mass gain from 33 PND, LH concentration, reduced testicular, pancreas, and liver mass, and increased epididymal mass ($P<0.05$). In tolerance tests, glucose intolerance increased at 60 PND and insulin resistance decreased. Treatment with Kp10 did not change the delay in vaginal opening and reduction in ovarian mass in F1 females caused by paternal hypothyroidism but led to a smaller gain in body mass in the second month of the offspring's life. Furthermore, it improved uterine morphology, pancreatic mass, and glucose tolerance at 60 PND. In F1 males, treatment with Kp10 normalized body mass gain at two months of age, as was also observed for plasma LH and testicular mass. Furthermore, it increased the mass of the vesicular gland and prevented the reduction in the height of the seminiferous epithelium caused by paternal hypothyroidism. In the glycemic metabolism of F1 males, Kp10 also improved glucose tolerance at 60 PND and further decreased insulin resistance. The findings of this study characterized the paternal effects of hypothyroidism and Kp10 treatment on the sexual development and glycemic metabolism of rat offspring. Here we show that paternal hypothyroidism in rats alters the reproductive and metabolic programming of offspring in a sex-dependent manner, and that paternal use of Kp10 is able to partially prevent these changes.

Keywords: thyroid; male; offspring development; kisspeptin.

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MALE REPRODUCTIVE BIOLOGY

Evaluation of *in vitro* pharmacological reactivity of the epididymal duct of adult rats exposed to aripiprazole

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Several studies have shown that the use of antipsychotics and antidepressants has a great impact on animal fertility. However, few studies has been done on the effect of third-generation medications, such as aripiprazole, on the male reproductive system. Studies carried out by our laboratory have demonstrated subtle changes in the sperm quality of adult rats exposed to the antipsychotic aripiprazole. Therefore, it is important to investigate the direct impact of exposure to different concentrations of aripiprazole on the contractility of the epididymal duct, with emphasis on evaluating whether there are effects on sperm transit time. Thus, the presente study aimed to investigate the possible effects of aripiprazole exposure on the contractility activity of the epididymal duct of adult Wistar rats. For this, epididymal ducts (n=4) collected from two animals without previous treatment were used. The study was approved by the Ethics Committee of UERN 006/21 and the Ethics Committee of UFERSA 23091.014948/2019-20. After euthanasia with CO2 and removal of the epididymis, the epididymal tail was isolated, unrolled and segmented into pieces measuring approximately 1.5 cm and placed in the organ bath. The tissues were then mounted in muscle chambers for digital recording of isometric tension development. All tissues were maintained under 1g of basal tension in Krebs nutrient solution with pH 7.4, at a constant temperature of 30ºC and continuously aerated with carbonic mixtures (95% O2 and 5% CO2). After 30 minutes of stabilization of the preparation, contraction to 80mM KCl was observed on two occasions separated by 30 minutes to assess tissue viability and stabilization of maximum tissue contraction. After 30 minutes of the last contraction to KCl, a concentration-response curve to norepinephrine was constructed, which was considered a control curve. After washing and relaxing the tissues (15 min), 10 µM aripiprazole was added for an incubation period (45 min) and then a new norepinephrine concentration-response curve was constructed in the presence of aripiprazole. The tissue was washed again and the process was repeated at concentrations of 300 and 1000 µM of aripiprazole. The concentrations necessary to promote 50% of the maximum contractile response (EC50) as well as the maximum contractile response (Emax) were obtained for each concentration. Our results showed that the plateau was not reached in the concentration-response curves to norepinephrine, which means that higher concentrations of norepinephrine should be used to reach receptor saturation. There was no alteration in the results of pEC50 (3.99+0.19 x 3.65+0.37 x 3.50+0.37 x 3.28+0.23) from 0, 10, 300 and 1000 µM of aripiprazole respectivelly ($p>0.05$). In the same way, there was no alteraion on the percentage of Emax(112.10+13.79 x 83.32+23.51 x 85.94+27.86) in the aripiprazole concentrations when compared to the control Emax (100.00 + 0.00).

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MALE REPRODUCTIVE BIOLOGY

Evaluation of the testicular histomorphometric of hyperglycemic rats treated with menthol

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Diabetes mellitus is a chronic disease characterized by a series of metabolic dysfunctions caused by hyperglycemia. Natural products with hypoglycemic activity have been studied for the treatment of diabetes such as menthol. Menthol has biological effects such as antiulcerogenic and antidiabetogenic properties, as described in the literature. Moreover, due to antioxidant activity of menthol, it can be used directly in cell culture. It is known that an increase in reactive oxygen species in the reproductive system of diabetic animals occurs , leading to problems in spermatogenesis. However, the possible beneficial effects of menthol on the male reproductive system of diabetic animals have not yet been investigated. For this, adult male Wistar rats were divided into five experimental groups (n=4-7/group): N group (normoglycemic group, treated with vehicle) and four hyperglycemic groups: H group (treated with vehicle), M group (treated with 50mg/kg of Menthol), G group (treated with 500mg/kg of glibenclamide) and MG group (treated with 50mg/kg of menthol + 500mg/kg of glibenclamide). Hyperglycemia in rats was promoted through a single dose of 45 mg/kg of streptozotocin (intraperitoneal injection). Rats were treated during 28 days. The body weight was recorded daily and the blood glucose was measured on days 1, 9, 18 and 28 of treatment. At the end of the treatment, the male rats were weighed and euthanized via intramuscular administration of ketamine (30 mg/kg) and intraperitoneal of thiobutabarbital (50 mg/kg). The right testes were fixed in bouin for histopathology and morphometric assays. Ethics Protocol number CEUA IBB/UNESP 9816221021 (ID 000322). Statistics: ANOVA and Tukey test ($p<0.05$). A decrease in weight gain was observed in animals from the hyperglycemic (33.60+10.42g) and menthol-treated (28.67+6.92g) groups compared to the normal group (81.10+6.88g) . Additionally, there was an increase in the relative weight of the testes in the groups treated with glibenclamide (5.68+0.29g) and menthol + glibenclamide (5.67+0.19g) when compared to the control group (4.25+0.21g). Finally, there was a decrease in the frequency of tubules in stages VII to VIII in the group treated with menthol + glibenclamide (17.60+2.04g) , when compared to the normal group (29.00+1.29g) . However, there were no alterations in the morphometric and histopathological evaluation in all treated groups. Thus, although a greater frequency of seminiferous tubules in stages VII and VIII may indicate greater spermatogenic activity, due to the other results, in the present experimental model, there is no testicular spermatogenic activity alteration.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Sperm quality reduction in rats subchronically treated with aripiprazole

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Depression is one of the most common psychiatric disorders worldwide and is a disease characterized by a dysfunction in the neurotransmission of serotonin (5-HT) and is treated through antidepressant medications. Aripiprazole, a third-generation drug, acts on serotonergic, noradrenergic and dopaminergic receptors and has been widely prescribed for the treatment of mental disorders. It is known that the male reproductive system, especially the epididymis, is highly innervated by the autonomic nervous system and drugs that act on neurotransmitters can directly impact sperm quality. However, studies have not yet been carried out on the possible influence of aripiprazole on male reproduction. Thus, the present study evaluated the effects of subchronic aripiprazole exposure on sperm quality in adult male Wistar rats. The study was approved by the Ethics Committee of UERN 006/21 and the Ethics Committee of UFERSA 23091.014948/2019-20. Twenty-one males (90 days old) were divided into 3 experimental groups (n=7/group): control group (Ctrl) that received vehicle solution (Dimethylsulfoxide – DMSO + saline solution), experimental 01 (Exp 1, received 3mg/kg of aripiprazole diluted in vehicle solution) and experimental 02 (Exp 2, received 6mg/kg of aripiprazole diluted in vehicle solution). The animals were orally treated for 28 days and then were euthanized to assess the sperm parameters. The right testes were used to determine the daily sperm production and the right epididymis were used to sperm collection to evaluate the sperm motility, morphology, concentration, and sperm transit time. Oral administration of aripiprazole caused a significant increase ($p<0.05$) in the absolute weight of the seminal gland in Exp 2 (0.96 ± 0.02g) when compared to the Control group (0.77 ± 0.03g) this increase it was also observed relative weight of seminal gland (mg/g) in the same Exp02 and Control groups (3.34 ± 0.09 X 2.79 ± 0.08). However, the weights of the other reproductive organs were similar between the groups ($p>0.05$). The daily sperm production and morphology were similar in all groups, but the progressive sperm motility was reduced ($p<0.05$) at the dosage of 3.0 mg/kg (Exp 01) when compared to the Ctrl group (57,7% x 37,7%). Thus, our results suggest that aripiprazole, although effective for the treatment of depressive and psychoactive diseases, exerts deleterious effects on reproductive parameters in this experimental model, suggesting an impact on sperm quality and, consequently, on fertility, which increases the concern regarding the indiscriminate use of such substances in doses higher than those used in the present study and taking into account the high reproductive efficiency of rodents, the impacts on humans may be even greater than those presented.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Skin-derived somatic cells of Antillean manatees (*Trichechus manatus manatus* Linnaeus, 1758) as a toxicity model for dimethyl sulfoxide

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The declining population of the Antillean manatee caused by ecosystem degradation and rising pollution has prompted interest in developing conservation strategies for this species (1). In this sense, germplasm cryopreservation has been an interesting tool for species conservation, aiming for their use in assisted reproduction techniques. However, the success of this tool depends on the combination of cryoprotectants used. Although dimethyl sulfoxide (DMSO) is used as a cryoprotectant in different cell types, its efficiency and cytotoxic effects have been related in a concentration-dependent manner. In aquatic mammals, the recovery of gametes is difficult and laborious. In contrast, skin samples are more easily to obtain and enable somatic cell recovery. Therefore, we evaluated if DMSO, in different concentrations (5, 8 and 10%), can induce apoptosis and oxidative stress on skin-derived cells from Antillean manatees, affecting its metabolism. All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 23091.010755/2019-32) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 71804-1). Then, four individuals, which were in the process of recovery and adaptation in the Advanced Base of the National Center for Research and Conservation of Aquatic Mammals of the ICMBio, were used. Somatic cells isolated from abdominal skin were cryopreserved in Dulbecco's modified minimal essential medium: F-12 nutrient mixture (DMEM/F-12) supplemented with fetal bovine serum (FBS) and DMSO in different combinations, resulting in three groups: (i) 5% DMSO and 95% FBS (5DMSO group), (ii) 8% DMSO and 92% FBS (8DMSO group), and (iii) 10% DMSO and 90% FBS (10DMSO group). Cells not subjected to cryopreservation were used as a control (control group). After warming, cells were cultured in DMEM/F-12 supplemented with 20% FBS and 2% antibiotic-antimycotic solution (38.5 °C and 6.5% CO2) until reaching 70% confluence. All cells were evaluated for apoptosis levels using the acridine orange and ethidium bromide, oxidative stress using the 2',7'-dichlorodihydrofluorescein diacetate probe for measurement of reactive oxygen species (ROS), and metabolism using 3-(4.5-dimethylthiazole-2yl)-2.5-diphenyl tetrazoline bromide. All data were expressed as mean ± standard error and analyzed by ANOVA followed by Tukey test ($P < 0.05$). Regarding apoptosis levels, no differences ($P > 0.05$) were observed between control and cryopreserved groups for percentages of viable cells (control: 93.8% ± 4.1, 5DMSO: 99.4% ± 0.3, 8DMSO: 99.2% ± 0.3, and 10DMSO: 98.9 ± 0.5), and necrotic cells (control: 1.3% ± 0.5, 5DMSO: 0.7% ± 0.3, 8DMSO: 0.9% ± 0.3, and 10DMSO: 1.2% ± 0.5). Moreover, values of ROS, evaluated in arbitrary fluorescence units (AFU), were similar ($P > 0.05$) between all groups (control: 1.0 AFU ± 0.5, 5DMSO: 1.2 AFU ± 0.7, 8DMSO: 1.2 AFU ± 0.6, and 10DMSO: 0.8 ± 0.5). Additionally, no differences ($P > 0.05$) were observed between control and cryopreserved groups for metabolism (control: 96.8% ± 3.2, 5DMSO: 95.4% ± 4.1, 8DMSO: 90.3% ± 9.7, and 10DMSO: 90.7% ± 8.6). These results indicate that DMSO, at 5%, 8% and 10%, does not alter metabolism, or induce apoptosis or increase oxidative stress in somatic cells derived from Antillean manatees. In summary, DMSO could be indicated as a cryoprotective agent for gametes of this species.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Canine testicular and epididymal proteomics signature

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To understand the physiological processes involved on spermatogenesis and sperm maturation, the aim of this study was described the proteome of the testis and canine epididymis. Testes and epididymides (caput, corpus and cauda) were collected from 3 dogs after orchiectomy. RIPA extraction buffer was added, and samples were sonicated, submitted to SDS-PAGE, tryptic digestion and mass spectrometry (nano-LC MS/MS). The variables were submitted to univariate and multivariate analysis using one-way ANOVA followed by Fisher's test, principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA) and variable importance in projection (VIP-score). Considering all tissues were identified 304 proteins. PCA identified 4 distinct groups. One-way ANOVA and VIP score identified 50 and 20 proteins with differential abundance ($\alpha > 1.5$), respectively. A total 18 proteins were found in both statistical analyses, 6 were increased in the corpus, 4 in the caput of the epididymis, 10 in the testis, but there was no protein in greater abundance in the cauda of the epididymis. Moreover, 8 proteins from 10 were in greater abundance in the testis and gradually decreased towards the epididymal cauda. The predominant biological process was related to cellular process in all tissues, following metabolic process molecular. Therefore, the main molecular functions were associated with binding and cargo receptor activity in testis, and binding and catalytic activity in epididymal compartments. The functions were mainly associated with heat-shock protection, metabolism, cellular respiration, and structural composition. We concluded that there is difference in abundance and function of the proteins in the 4 compartments and this is related to the spermatogenesis and sperm maturation, and the heat-shock proteins family were main proteins found in the testes. These proteins must be studied to search for a antigen to produce a contraceptive vaccine.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Cryopreservation of collared peccary semen (Pecari tajacu) previously cooled and stored in BotuTAINER® at 5 °C for up 48 hours

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Given the ongoing advancements in the development and application of assisted reproduction techniques for collared peccaries, there is a need to enhance semen processing methods. This is crucial in seeking effective approaches to preserve sperm and facilitate the exchange of genetic material within different regions. Cryopreservation stands out as the primary method for preserving semen over an indefinite period; however, this technique encounters several limitations in field conditions, primarily concerning the transportation of the material. Against this backdrop, the aim of this study was to verify the possibility of cooling storage of peccary semen for 4, 24 and 48 hours prior to cryopreservation, using Tris + egg yolk (20%) extender, in order to maintain post-thaw kinetic and functional aspects. For this purpose, ejaculates from ten adult males from the Wild Animal Multiplication Center (CEMAS - UFERSA, Mossoró, RN, Brazil) were used. The ejaculates were obtained by electroejaculation through a protocol previously defined for the species, and carried out under anesthesia with propofol (5 mg/kg IV) in bolus. The samples were separated according to storage time intervals (4, 24, and 48 hours) and diluted in Tris + egg yolk (20%) for cooling followed by cryopreservation with a final concentration of 3% glycerol in the extender. The control group was stored in a biological incubator at 5 °C for 4 hours and the remaining groups were stored in a transport container (Botutainer®, Botupharma, Botucatu, Brazil) at 5 °C for 24 and 48 hours. All the samples were assessed for total and progressive motility, membrane functionality and integrity, mitochondrial activity, and the number of sperm attached to the hen's egg perivitelline membrane. The results were expressed as mean and standard error (SEM). Residual normality was assessed using the Shapiro-Wilk test, and variance homogeneity was verified through the Bartlett test ($P < 0,05$). After thawing, no statistical differences were observed for total and progressive motility, which presented the respective values for the storage-time intervals of 4 hours (45.8 ± 7.9 and 27.0 ± 5.8), 24 hours (43.0 ± 9.1 and 20.4 ± 5.1), and 48 hours (44.4 ± 6.5 and 20.6 ± 4.1). Membrane functionality and integrity, as well as mitochondrial activity, also showed no statistical difference. The samples stored for 4 hours (124,8 ± 22,2) showed lower ($P < 0.05$) membrane-bound sperm potential when compared to the fresh control (256,5 ± 12,1), but did not differ from the other treatments 24h (213,9 ± 48,1) and 48h (254,6 ± 27,8). The cooling of samples for up to 48 hours in a transport container, diluted in Tris + egg yolk (20%), proved to be a viable alternative before cryopreservation, enabling the initial processing of samples in remote or hard-to-reach areas. Tris acts as a pH regulator for semen, and egg yolk serves as a cryoprotectant, preventing damage to the spermatozoa's plasma membrane and functional integrity during cooling and cryopreservation. Storage in the incubator for 4 hours exhibits a faster cooling curve until stabilization at 5 °C when compared to storage in a Botutainer® for 24 and 48 hours, whose manufacturer states it takes around 5 hours to reach 5 °C and stabilize. The observed difference in membrane binding potential can be explained by the variation in cooling curves, where the slower curve could potentially ensure the maintenance of sperm binding potential. In conclusion, collared peccary semen can be effectively cryopreserved following dilution in Tris + egg yolk (20%) extender and cooling storage for up 48 hours in a transport container (Botutainer®) at 5 °C.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Histological assessment of tissue damage in bovine testicles: intrarater and interrater reliability

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Cryopreservation is a method used for fertility preservation in many situations, like man undergoing gonadotoxic treatments, species at risk of extinction or animals of high zootechnical value that die early. Despite cryopreservation being a valid solution to address issues such as infertility, the tissue and cells can suffer damage during the cryopreservation process. For evaluation of the success of the cryopreservation histological evaluations of testicular tissue are used to analyze integrity of morphology and functionality. The most commonly used is the classical histology Hematoxylin and Eosin (H&E) which allows the morphology of testicular tissue. Although classifications have included parameters and scores, histological evaluations remain complex and time-consuming due to the high complexity of testicular tissue morphology and the various cell types present, resulting from various stages of spermatogenesis. Previously literature contained parameters and scores for morphological assessments related to the absence of clear standardization and the complexities in reproducibility of scores. These issues gain high significance in search and clinical contexts where precision of assessments interferes with the results. This study has an objective to standardize a scoring table that encompasses the most important characteristics to assess the quality of testicular tissue after the cryopreservation process. The study follows the design for histological evaluation and selected three raters (undergraduate student, a PhD student and a master student). Three pairs of testicles from adult bovines were collected and fragments of tissues were exposed to vitrification. Next the process, the fragments underwent histological processing and the morphological parameters were carefully analyzed through a preliminary analysis of the seminiferous tubules. The 60 photomicrographs of seminiferous tubules were available to the evaluators in two different times. They were categorized into a scoring table with descriptions as alterations in the basal membrane, epithelial organization, spaces between (Gap) epithelial cells, retraction of the seminiferous epithelium, nuclei condensation, loss of seminiferous tubule cells, and scored between 0 and 3. As data (measurement 1, measurement 2, and averaged measurements scores) were analyzed by intraclass correlation coefficients (ICC). For rater 1 ranged from 0.000 (poor) for the pyknotic nuclei to 0.608 (goof) for the loss epithelial cells in the histological evaluation of the fresh testicular bovine tissue and ranged from 0.000 (poor) for the pyknotic nuclei to 0.875 (excellent) for the retraction of the seminiferous epithelium in the histological evaluation of the vitrified testicular bovine tissue. The ICC for rater 2 ranged from 0.103 (poor) for the loss epithelial cells to 0.574 (fair) for the pyknotic nuclei in the histological evaluation of the fresh testicular bovine tissue and ranged from 0.584 (fair) for the pyknotic nuclei to 0.869 (excellent) for the retraction of the seminiferous epithelium in the histological evaluation of the vitrified testicular bovine tissue. The ICC for rater 3 ranged from 0.000 (poor) for the retraction of the seminiferous epithelium to 0.851 (excellent) for the epithelium organization in the histological evaluation of the fresh testicular bovine tissue and ranged from 0.227 (poor) for the pyknotic nuclei to 0.595 (fair) for the spaces between epithelium cells in the histological evaluation of the vitrified testicular bovine tissue. For the first measurement, reliability indices showed variations among the raters, while in the second assessment, reliability was higher. This factor may have been influenced by training between the first and second assessments with the table. Another factor that may have influenced the reliability indices was the quality of the tissue after vitrification, in addition to histological processing. Thus, the table needs to be adjusted regarding the description of the characteristics, avoiding the subjectivity of the descriptions.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Use of different closed systems for the vitrification of testicular tissue from prepubertal dogs

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Over the years, important methods of conserving male genetic material based on sperm storage have been promoted for different species, including dogs. Recently, however, the conservation of testicular tissue has become an alternative for situations in which canines with valuable genotypes die suddenly. In this context, the objective of this study was to evaluate the effects of different closed vitrification systems on the testicular tissue of pre-pubertal dogs. The pair of gonads of five dogs, aging from 3 to 6 months old, were surgically collected, washed in saline solution, and fragmented (1 a 3 mm3). Six fragments were immediately fixed in bouin, for morphological analysis using hematoxylin-eosin staining, and 4% paraformaldehyde, to assess DNA integrity, constituting the control group. Other 32 fragments were subjected to the equilibrium solution (SE) consisting of Dubelco`s Modified Eagle Medium (DMEM) with 20% fetal bovine serum, 0.75% dimethyl sulfoxide (DMSO), 0.75% ethylene glycol (EG) for 10 min. Then, fragments were immersed in the vitrification solution (SV), which was similar to SE but with twice cryoprotectants concentration, for 5 min. Samples were then vitrified using two different closed systems: the first was the Ovarian Tissue Cryosystem (OTC), in which the fragments were exposed to the two solutions inside the device (1); in the other, vitrification was conducted in cryotubes (CV), in which the exposure was carried out in culture plates and then the fragments were transferred to cryotubes (2). Both systems were closed, immersed in nitrogen liquid and stored for one week. After this period, the samples were warmed using solutions containing decreasing concentrations of sucrose, to remove the cryoprotectants. Fresh and vitrified samples were evaluated for viability by vital fluorescent probes (Hoechst 33358 and propidium iodide), histomorphology according to scores (3 – adequate; 2 regular; 1 – poor) using the following criteria (3): separation of the basal membrane, structure integrity, cell swelling, cell loss and rupture, and DNA integrity using the terminal deoxynucleotidyl transferase dutp nick-end labeling (TUNEL). Data were expressed as mean ± standard error and evaluated by ANOVA followed by Fisher's test for viability and Kruskal-Wallis for morphology and DNA integrity ($P<0.05$). After heating, all samples subjected to the vitrification procedure suffered a significant reduction in viability when compared to the fresh group (82.8 ± 3.4%). However, CV (41.8 ± 5.4%) showed greater ($P < 0.05$) viability than OTC (35.5 ± 7.5%). Regarding morphological scores, all vitrification methods were efficient in preserving the integrity of the basement membrane, presenting retraction rates (OTC: 2.95 ± 0.01; CV: 2.94 ± 0.01) and rupture of membrane (OTC: 2.94 ± 0.02; CV: 2.92 ± 0.02) similar to the fresh group (2.97 ± 0.01 and 2.93 ± 0.02). For cellular distinction, nuclear visualization, nuclear condensation and tubule structure, there was a significant reduction in both vitrified groups, when compared to the fresh samples($P<0.05$). Regarding such parameters, there were no significant differences regardless the closed vitrification system used, but a better preservation ($P < 0.05$) of nuclear condensation was observed for OTC (2.06 ± 0.04), which presented a low rate of pyknotic nuclei, in comparison to CV (1.86 ± 0.02). Additionally, DNA integrity was better preserved by CV (95.7 ± 1.23), being like the non-cryopreserved group (99.0 ± 0.57), whereas OTC (90.8 ± 1.92) significantly affected this parameter ($P < 0.05$), presenting a higher rate of DNA damage. In conclusion, we suggest the use of the vitrification in cryotubes (VC) as an adequate closed system for the preservation of morphological parameters and DNA integrity of testicular tissue from pre-pubertal dogs.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Effects of Menthol Exposure on Sperm Quality in Hyperglycemic Rats

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It is known that diabetes mellitus has a major impact on spermatogenesis, mainly due to oxidative stress promoted by reactive oxigen species (ROS) on germ cells. Furthermore, there are several studies showing the impact of diabetes on the sperm maturation process, thus interfering with male fertility. On the other hand, products of natural origin have been widely used in order to mitigate the effects of oxidative stress on the reproductive system. Menthol, whose anti-inflammatory and antioxidant activity is already well described for several systems, has not yet been evaluated for its potential beneficial effect on sperm quality in diabetic rats. Thus, the present study aimed to evaluate the effects of menthol exposure on sperm quality in hyperglycemic rats. Adult male Wistar rats were divided into five experimental groups (n=4-7/group): normoglycemic group (treated with vehicle), hyperglycemic (treated with vehicle), menthol group (treated with 50mg/kg of Menthol), glibenclamide group (treated with 500mg/kg of glibenclamide) and menthol + glibenclamide group (treated with 50mg/kg of menthol + 500mg/kg of glibenclamide). Hyperglycemia in rats was promoted through a single dose of 45 mg/kg of streptozotocin (intraperitoneal injection). Rats were treated during 28 days. At the end of the treatment, the male rats were weighed and euthanized via intramuscular administration of ketamine (30 mg/kg) and intraperitoneal of thiobutabarbital (50 mg/kg). The left testis and epididymis, seminal vesicle (with and without its fluid) and ventral prostate were dissected and weighed. This testis was used for sperm counts. The epididymal cauda was used for sperm collection to sperm quality assay (motility, morphology and count). Ethics Protocol number CEUA IBB/UNESP 9816221021 (ID 000322). Statistics: ANOVA and Tukey test ($p<0.05$). A decrease in sperm concentration (x106) was observed in the epididymis in the menthol + glibenclamide group (122.1 + 3.9) compared to the normoglycemic group (297.0 + 33.0) and hyperglycemic group (280.5 + 57.1), respectivelly. Additionally, an acceleration of transit time (in days) was observed in the menthol + glibenclamide group (2.5 + 0.2) compared to the normoglycemic group (6.8 + 0.5). Finally, there was a decrease in the percentage of sperm with progressive motility in the menthol + glibenclamide group (35.2 + 4.7) compared to the normoglycemic (65.4 + 3.9) and glibenclamide (60.9 + 5.4) groups. Thus, although in the literature hyperglycemia directly affects sperm quality and spermatogenesis, in the present study, only the combined treatment of menthol + glibenclamide impacted sperm quality. Further studies should be conducted to better explain the potential combined effects of menthol.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

The conditioned medium derived from human Wharton's jelly cells enhances the fertilization potential of murine sperm

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Several external factors inherent to lifestyles can directly interfere with the maturation process of male gametes, spermatozoa (1). In this way, in vitro culture emerges as an excellent alternative, as it mimics the in vivo environment, allowing the use of different substances that can enhance the maturation and capacitation of these gametes. In this context, the conditioned medium produced by mesenchymal stem cells derived from Wharton's jelly may be a promising alternative, as these cells secrete a range of molecules that can enhance in vitro development (2). Therefore, the aim of this study is to assess the fertilization potential of Wistar rat spermatozoa after incubation in conditioned medium from human Wharton's jelly cells. To this, Wharton's jelly fragments were dissected and cultured in vitro for 72 hours to produce the conditioned medium. Murine spermatozoa were collected from the epididymis tail and distributed in Eppendorf tubes at a concentration of 2x105, subsequently incubated. The incubation medium consisted of α-MEM supplemented with 1.25 mg of bovine serum albumin (α-MEM+), using different proportions of conditioned medium (25, 50, 75, 100%) for a final volume of 1 mL. Incubation was carried out at 37°C in a humidified atmosphere with 5% CO2 for 1 hour. For the analysis fertilization potential of the spermatozoa, the chicken egg perivitelline membrane penetration test was perfomed. The internal membrane was removed, isolated, and fragmented (1x1x1 cm). After the incubation period, the spermatozoa were recovered and then incubated with the membrane in α-MEM+ under the same conditions described above for 30 minutes. Subsequently, the membranes were washed in 0.9% saline solution to remove non-adherent spermatozoa and placed on histological slides for spermatozoa quantification. All spermatozoa present in 6 randomly fields were quantified. Data were expressed as mean ± S.E.M, and the Shapiro-Wilk normality test was performed. Regarding variance, one-way ANOVA with Tukey's post hoc test was conducted using GraphPad Prism 9.0 software (Graphpad Software, Inc., San Diego, USA). As a result, it was observed that spermatozoa incubated in 100% conditioned medium showed a higher fertilization capabitily compared to all other treatments ($P<0.05$). This increase in the fertilization potential of the spermatozoa may be attributed to the various beneficial substances that Wharton's jelly cells secrete, such as cytokines and growth factors that are possibly present in the conditioned medium, such as vascular endothelial growth factor (VEGF), interleukin 6 (IL-6), and anti-apoptotic proteins like Bcl-xL, Bcl-2 (2). In summary, the use of conditioned medium at a concentration of 100% increased fertilization capability of murine spermatozoa, constituting an alternative for infertility or subfertility treatments.

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Animal Reproduction

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MALE REPRODUCTIVE BIOLOGY

Testicular histomorphometric evaluation from adult male rats exposed to aripiprazole

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Depression is one of the most common psychiatric disorders worldwide and affects children, adults and the elderly. It is a pathological condition whose treatment is through the intervention of psychotropic drugs available, including aripiprazole. Aripiprazole, a drug that has high affinity for serotonin, dopamine and noradrenaline receptors for the treatment of mental disorders, mainly schizophrenia and depression. Therefore, some side effects of these drugs are still unknown, mainly linked to the reproductive system. Male reproductive physiology is a system that is based on the hypothalamic-pituitary-gonad axis, steroid-dependent and any compound that acts on the control of the axis interferes with the normal development of the male genital tract. Thus, the present study evaluated the histopathology and morphometric of the testis from adult male Wistar rats exposed subchroniclly to aripiprazole. The study was approved by the Ethics Committee of UERN 006/21 and the Ethics Committee of UFERSA 23091.014948/2019-20. The experiment used 21 males (90 days old) divided into 3 experimental groups: control group (Ctrl - vehicle), 3mg/kg (Exp 1) and 6mg/kg (Exp 2) of aripiprazole diluted in vehicle solution. The drug was administered orally (gavage) and the experimental groups received the drug diluted in vehicle (Dimethylsulfoxide – DMSO + saline solution). After 28 days of treatment, the 21 males (n=7/group) were euthanized to collect the organs. The right testis, epididymis, vas deferens, ventral prostates and seminal glands were removed and their weights were determined. The left testis was used for morphometric and histopathological analysis under an optical microscope. For histopathology, 100 sections of the seminiferous tubules of each animal were analyzed and observing the appearance of the epithelium, content of the lumen and interstitium of the testis and possible morphological lesions. The seminiferous tubules and the appearance of the testicular interstitium were also evaluated, with emphasis on Leydig cells. Morphometric analyzes were performed using 10 seminiferous tubules from each animal in stage IX of spermatogenesis and the diameter and height of the germinal epithelium were obtained. Furthermore, Leydig cell volume and Sertoli cell number were determined. Our results showed that there was a significant increase in the diameter of the seminiferous tubules in stage IX for the 3.0 mg/kg group (321.10µm + 5.76 µm) when compared to the control (285.10 µm + 5.93 µm) and to the 6.0 mg/kg group (294.50 µm + 6.09 µm), $P < 0.05$. There was no difference between groups for epithelial height, Leydig cell volume and Sertoli cell count ($P > 0.05$). Regarding histopathological evaluation, there was also no significant difference between the groups evaluated, $P > 0.05$. Histopathological evaluation is generally accepted as the most sensitive parameter to detect effects of chemical substances on male reproductive function, our data demonstrate, at least in this experimental model, that exposure to aripiprazole did not directly affect the animals spermatogenesis.

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MALE REPRODUCTIVE BIOLOGY

Impacts on fertility and sexual behavior of animals treated with aripiprazole

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The serotonergic system can directly influence the reproductive function of vertebrates, due to its communication with the sexual steroid system. Currently, drug therapies for psychiatric problems generate secondary effects on reproduction and consequently fertility as they can directly interfere with the animals' libido. Among the most used new drugs are third-generation antipsychotics, such as Aripiprazole. Studies carried out in our laboratory have shown an impact on the sperm quality of males exposed to doses during subchronic treatment. However, direct evaluations of the possible influence of this drug on the sexual behavior and fertility of male Wistar rats had not yet been investigated. Thus, the present study aimed to evaluate the sexual behavior and fertility of adult male rats exposed to the antipsychotic aripiprazole. To this end, male rats (n=5/group) were divided into three experimental groups: control group (received vehicle solution - Dimethylsulfoxide + saline) experimental 01 group (3mg/kg of aripiprazole diluted in vehicle solution) and experimental 02 group (6mg/kg of aripiprazole diluted in vehicle solution) and treated for 28 consecutive days. At the end of the treatment, the animals were placed to perform sexual behavior with nulliparous females in a 1:1 ratio during 40 min of the dark cycle of the light/dark cycle photoperiod. The study was approved by the Ethics Committee of UERN 006/21 and the Ethics Committee of UFERSA 23091.014948/2019-20. The following parameters were observed: latency of first mount, latency of first intromission and latency of first ejaculation. In addition, the number of mounts, intromissions and ejaculations were evaluated. At the end of the sexual behavior test, females with positive sperm were considered pregnant and were maintained for 20 days. At day 20, the females were euthanized by saturation anesthetic (xylazine and ketamine) and laparotomy was performed. The pregnant uterus and ovaries were collected, weighed and evaluated. Fetal and placental weights, the number of corpora lutea, live and dead fetuses were also evaluated and pre- and post-implantation loss rates, the proportion of male and female fetuses, as well as the fertile potential of each experimental group were calculated. The results of the sexual behavior tests showed that there was no statistical difference in relation to the latency parameters. However the number of mounts was significantly lower ($P < 0.05$) at the dosage of 3.0 mg/kg (6.0+1 .5), when compared with the control group (16.00 + 3.24). Furthermore, regarding the fertility test, a significant reduction ($P < 0.05$) in the fertile potential of males in the 6.0 mg/kg group (83.00%) was observed when compared to the control group. (93.00%). Considering that psychoactive drugs can impact neuronal control, including sexual behavior, it can be stated that exposure to aripiprazole, in this experimental model, impacted the animals' libido and consequently their fertility potential. More studies must be carried out to evaluate how much this reduction could be directly linked to the sperm quality of the animals or to the control of the hypothalamic-pituitary-gonad axis, mainly related to sexual desire.

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MALE REPRODUCTIVE BIOLOGY

BOVINE TESTICULAR TISSUE CRYOPRESERVATION: AN INTEGRATIVE LITERATURE REVIEW

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The cryopreservation of bovine testicular tissue emerges as a viable alternative for preserving reproductive potential (1). In addition to allowing the storage of tissue fragments containing a large number of germ cells, it also preserves the niche around these cells, providing physical support and regulatory mechanisms for spermatogonial stem cells (2). The aim of this study was to analyze scientific literature related to protocols for the cryopreservation of bovine testicular tissue. This is an integrative literature review conducted in March 2023. The PICO strategy was used to formulate the guiding question: "What scientific evidence is available in the literature regarding the cryopreservation of bovine testicular tissue?" The databases consulted were PubMed, Scopus, and Web of Science, using the descriptors "cryopreservation," "tissues," "tissue," "testis," "testicle," and "cattle." Articles available in full and without language and publication date restrictions were selected. Review studies and duplicates were excluded. 1.273 publications were located, with 663 in PubMed, 33 in Scopus, and 577 in Web of Science. After reading the abstracts of these works and considering the inclusion and exclusion criteria, 12 articles were selected. Regarding the publication year, it is noteworthy that between 2015 and 2016, three and two studies were published, respectively. Ten studies were conducted in China, five studies were published in the journal Cryobiology, and three in Andrologia. The age of the animals ranged from 1 day to 4 years. The most commonly used cryopreservation medium was Modified Eagle's Medium Dulbecco's, employed in eight studies. All studies utilized the technique of uncontrolled slow freezing. The sizes of testicular tissue fragments and storage times varied from 0.3 cm^3 to 2 mm^3 and from 7 days to 1 year, respectively. Regarding cryoprotectants, dimethyl sulfoxide (DMSO) was the most common, used in eleven studies, followed by glycerol in four studies, and ethylene glycol, trehalose, and propylene glycol in three studies. The results indicated that tissues cryopreserved with 10% DMSO showed higher cellular viability. Furthermore, protection of structural integrity and continuity of cellular function, as well as expression of the genes CREM, Stra8, and HSP70-2, related to spermatogenesis activation, were observed. Supplementation of trehalose to the cryopreservation medium demonstrated a higher percentage of cellular viability, enzymatic activity, and antioxidant activity. It is concluded that the standardization of protocols for cryopreservation of bovine testicular tissue still remains a challenge to be overcome due to the difficulty of harmonizing different protocols, in addition to factors such as exposure time and cryoprotectant solution.

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MALE REPRODUCTIVE BIOLOGY

Comparison between slow freezing and solid surface vitrification methods on testicular tissue cryopreservation from prepubertal collared peccaries

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Cryopreservation of male gonadal tissues has been rising as a promising alternative for the conservation of genetic material, adding to the preservation of sperm in the formation of biobanks, and contributing to the maintenance of genetic variability for wild populations. For this application, however, it is necessary to determine the most appropriate protocols for processing testicular tissues, respecting the histological characteristics of the different species, as well as the peculiarities inherent to the age of the donor individuals. Therefore, the aim was to establish an efficient protocol for the cryopreservation of testicular tissues from prepubertal collared peccaries (Pecari tajacu). For this purpose, we used 5 prepubertal animals from the UFERSA Center for Wild Animals Multiplication (CEUA no. 23091.004271/2014-71). Five pairs of testicles were fragmented (3 mm³) and allocated in non-cryopreserved (control) and cryopreserved groups following three different freezing protocols: slow freezing (SF), solid surface vitrification with 3M cryoprotectant (SSV 3M) and solid surface vitrification plus 6M cryoprotectant (SSV 6M). The same cryopreservation solution composed for Minimum Essential Medium (MEM) supplemented with 0.25 M sucrose, and 10% (v/v) fetal bovine serum (FBS), and different concentrations of intracellular cryoprotectants was used to all protocols. For SF, a 2mL cryopreservation solution plus combination of 0.75 M dimethyl sulfoxide (DMSO) and 0.75 M ethylene glycol (EG) was used, and tissue fragments were placed into a Nalgene (Mr. Frosty, Fisher Scientific) freezing container with isopropyl alcohol at room temperature that then was transferred into a -80°C freezer for 12 hours and stored in cryobiological cylinder. For SSV, fragments were separated into two groups, in which first was immersed in cryopreservation solution plus 3M cryoprotectant (1.5 M DMSO and 1.5 M EG) and the second group was initially immersed to that same solution, and then to a similar solution plus 6M cryoprotectant (3.0 M DMSO and 3.0 M EG). Then, the remaining solution was removed from fragments with aseptic absorbent filter, and placed for 30 seconds on an aluminum sheet as a solid surface of high conductivity in contact with nitrogen, then they were placed in cryotubes and stored. After one week, the cryovials were maintained for 1 min at 25 °C and immersed in a water bath at 37 °C for 30 sec. For removal of cryoprotectants, all fragments were washed three times for 5 min in MEM, 10% FBS, with decreasing concentrations of sucrose (0.50, 0.25 M, and without sucrose). All samples were evaluated for viability by vital fluorescent probes (Hoechst 33358 and propidium iodide), histomorphology according to scores (3 – adequate; 2 regular; 1 – poor) using the following criteria: separation of the basal membrane, structure integrity, cell swelling, cell loss and rupture, and DNA integrity using the terminal deoxynucleotidyl transferase dutp nick-end labeling (TUNEL). Data were expressed as means ± standard error. Viability and DNA integrity was compared using Tukey and morphology by Kruskal-Wallis. Differences were considered significant when $P < 0.05$. All treatments as SF (57.0 ± 3.9%), SSV 3M (59.2 ± 4.4%), and SSV 6M (56.4 ± 3.5%) provoked a decrease ($P < 0.05$) in viability when compared to the fresh samples (88.8 ± 1.93%). Regarding histomorphology, all the criteria analyzed were affected by cryopreservation, regardless the protocol used ($P < 0.05$); however, SF (2.08 ± 0.05 and 2.33 ± 0.07) and SSV 6M (1.93 ± 0.04 and 2.30 ± 0.07) provided a most efficient preservation ($P < 0.05$) of the structure integrity and cell rupture than SSV 3M (1.87 ± 0.05 and 2.08 ± 0.06). For the cell swelling, the SF (2.16 ± 0.06) and SSV 6M (2.13 ± 0.04) presented similar scores and both were better than SSV 3M (1.92 ± 0.05). For cell loss, SSV 6M (2.60 ± 0.05) was similar to SF (2.47 ± 0.07), and better SSV 3M (2.33 ± 0.07). The DNA integrity was conserved only by SSV 6M (97.0 ± 0.68), which was similar to the fresh control (99.4 ± 0.28). These preliminary results suggest that the combination SSV 6M is recommended for the cryopreservation of the testicular tissues from prepubertal collared peccaries.

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MALE REPRODUCTIVE BIOLOGY

Male pseudohermaphroditism with mosaicism in a dog: a case study by genotyping and karyotyping

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Intersexuality and pseudohermaphroditism in dogs are congenital disorders in the development of sexual organs, leading to gonadal and phenotypic variations of the genital tract, as opposed to characteristics determined by chromosomal sex. This study aims to report the occurrence of sexual ambiguity in a dog and its respective etiological and clinical-surgical management. A dog of approximately 3 years of age, mixed breed, was admitted to the Veterinary Hospital of FMVZ-USP with a complaint of exposure of a structure similar to the penis, with signs of trauma to the local mucosa and pinpoint bleeding. Due to being a semi-domesticated animal, there was no precise history available. Clinical evaluation revealed a complete penile structure, but with perineal insertion, directed caudally, and surrounded by a skin extension with an ambiguous appearance of large vulvar lips or preputial hypoplasia. There was penile hypospadias, with the urethral meatus positioned ventrally on the penile body. In the scrotal topography, there was a skin fold resembling the scrotum, but in the absence of testicles. Abdominal ultrasonography revealed ellipsoid structures with preserved shape and irregular contours (3.75 cm length x 2.34 cm width x 3 cm thickness), resembling ectopic testicles, with coarse echotexture and increased echogenicity. In close contact with the dorsal wall of the prostate, there was a structure with defined and regular contours, filled with anechoic high-cellularity content with an approximate volume of 7 mL, measuring 4.84 cm in its longer axis. The prostatic urethra occupied the usual topography, but with thickened (0.19 cm) and echogenic walls. A 3 mL blood sample was collected using a heparinized syringe (0.1 mL of sodium heparin) for leukocyte culture to establish cytogenetic analysis. With the clinical confirmation of genital malformation, the animal underwent exploratory laparotomy, revealing structures morphologically compatible with testicles and epididymis caudal to the kidneys, connected by a tubular structure macroscopically resembling uterine horns. The excision of such structures was performed and sent for histopathological analysis, along with a tissue fragment submitted for genotyping. For the external genitalia, penectomy was performed, followed by reinsertion of the urethra into the lateral mucosa, remaining covered by a skin extension in the perineal region, similar to vulvar lips. The postoperative period was uneventful, and the animal was discharged after verifying the absence of urethral stenosis, dysuria, or stranguria. Histopathological examination revealed the presence of gonads morphologically compatible with testicles and epididymis, but presenting testicular hypoplasia and bilateral epididymal dysplasia. On the other hand, the tubular structure resembling the uterus presented hypoplastic endometrium and chronic metritis, suggesting a genital configuration compatible with intersex. After counting 50 metaphases in the cytogenetic analysis, it was identified that 62% had 2n=78; 12% had 2n=79; 8% had 2n=75; 2n=74, 76, and 77 were found in 4% of the metaphases of each diploid number; 2n=62, 72, and 73 were found in 2% of each diploid number. Thus, the modal number was 2n=78, and the variation in the diploid number occurred because of the addition (2n=79) or absence of acrocentric chromosomes (2n<78). The karyotype with 2n=78 was composed of 76 acrocentric autosomes ranging from large to small, a large submetacentric X chromosome, and the Y was a tiny metacentric - smallest chromosome of the diploid complement. Sex genotyping of the tissue was performed by PCR of the AMEL gene, based on the length polymorphism between the X and Y chromosomes. Intersex is a complex sexual development anomaly, both for diagnosis and therapeutic purposes, due to multiple manifestations. This study reveals the existence of genital malformation, with phenotypic sex ambiguity but with confirmation of male genetic sex, as well as the existence of autosomal mosaicism in cytogenetic analysis. Thus, this case contributes to the understanding of congenital genital disorders and the categorization of chromosomal abnormalities.

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MALE REPRODUCTIVE BIOLOGY

EFFECT OF ADDING SODIUM SELENITE TO TRIS-YOLK DILUENT ON PLASMA AND ACROSOMAL MEMBRANE INTEGRITY OF FROZEN-THAWED SHEEP SEMEN

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Cryopreservation offers numerous advantages in preserving sheep semen, but oxidative stress can irreversibly damage sperm cells (1). Thus, it is crucial to find substances with cryoprotective and antioxidant properties to neutralize oxidants during preservation (2). Sodium selenite (SS) emerges as a potential antioxidant additive due to its association with selenoproteins, such as glutathione peroxidase (GPx) (3). Therefore, the objective of this study was to evaluate the efficiency of adding SS to TRIS-based extender with egg yolk in the cryopreservation of semen from Santa Inês sheep. For this, 10 ejaculates were collected from three rams (10 ejaculates/ram) using an artificial vagina. The ejaculates were diluted with TRIS-egg yolk and organized into the following treatments: CON Group, in which the diluent did not contain SS; and the SS2, SS4, and SS6 groups, in which 2 µg/mL, 4 µg/mL, and 6 µg/mL of SS were added to the diluent, respectively. After addition, the diluted samples were placed in a cold chamber at 5°C for 2 h. Then, the samples were placed in 0.5 mL straws, sealed, placed for 15 minutes under nitrogen vapor at 5 cm from the liquid slide, and finally immersed in liquid nitrogen. Thawing was performed at 37°C for 30s. The samples were analyzed for sperm motility, plasma membrane and acrosomal integrity, mitochondrial activity, quantification of oxidative stress, and formation of cleaved structures after in vitro fertilization of oocytes. Results are expressed as mean ± standard error ($P < 0.05$), with ANOVA followed by Tukey's test used, using R software version 4.2.0. The addition of 2 µg/mL of SS increased VAP, VCL, STR, and ALH parameters and acrosomal integrity compared to other treatments ($P<0.05$). Regarding mitochondrial activity, SS0 and SS2 were more effective than SS4 and SS6 ($P<0.05$). SS4 increased the percentage of active sperm, while SS6 resulted in more inactive cells ($P<0.05$). Additionally, 4 µg/mL of sodium selenite reduced TBARS levels compared to the control group ($P<0.05$), possibly due to increased GSH-Px enzyme activity. In in vitro fertilization, SS6 had a cleaved structure rate similar to the control but higher than SS2 and SS4. Regarding the number of blastomeres, SS4 and SS6 were similar, with only SS4 being superior to CON and SS2. There was no statistical difference for 2 and 8 blastomeres. Sodium selenite also acts by inhibiting apoptosis in oocytes, improving their quality, and contributing to healthier embryonic development after fertilization (4). In conclusion, the inclusion of sodium selenite benefits the mitochondrial activity of cryopreserved ram semen, although further research is needed to determine the ideal concentration for sheep semen preservation.

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MALE REPRODUCTIVE BIOLOGY

Ultrasonographic evaluation and testicular echobiometry in oncilla Leopardus tigrinus

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Despite the variable geographical distribution, ranging from the mountain forests in Costa Rica to the semiarid biome of the Brazilian Caatinga, the oncilla Leopardus tigrinus (Schreber, 1775) is a small neotropical felid classified as ecologically vulnerable by the International Union for Conservation of Nature. The species faces threats such as habitat fragmentation, hunting, and illegal trade, emphasizing the importance of understanding the reproductive morphophysiology for adopting appropriate management and conservation strategies. This work aimed to evaluate the ultrasonographic characteristics and echobiometry of the testes of L. tigrinus in the Caatinga biome. Procedures were approved by CEUA-UFERSA No. 20/2023 and SISBIO No. 88201-1. For this study, three adult animals, with an average age of 2.17 ± 0.44 years and weight of 2.53 ± 0.21 kilograms, housed in zoos in Fortaleza, CE (Latitude: 3° 43' 6" S, Longitude: 38° 32' 36" W) and Canindé, CE (Latitude: 4° 21' 29" S, Longitude: 39° 18' 7" W) were used. All animals were undergone a physical examination to ensure that they were in good health, free from diseases and suitable for the anesthetic procedure for chemical restraint. Animals were captured from their enclosures using a net and chemically restrained with a combination of dexmedetomidine and ketamine hydrochloride. Oncillas were positioned in dorsal decubitus, and acoustic gel was applied in the scrotum area. Ultrasonographic characteristics were evaluated using a portable device with an 8 MHz linear frequency transducer, in B-mode and real-time (Vinno A5, Vinno Technology Co. Ltd., Suzhou, Jiangsu, China). The testicular parenchyma was assessed for echogenicity and homogeneity. Sagittal and cross-sectional images were obtained to measure length, height, and width (centimeters) between the borders of the right (RT) and left (LT) testicles, using electronic cursors integrated with the ultrasound machine (1). The dimensions were applied to calculate the testicular volume using Lambert's formula (2). For descriptive analysis, the Statview 5.0 software (SAS Institute Inc., Cary, NC, USA) was used. The results were expressed as mean ± standard error. In the cross-sectional images, it was possible to clearly identify a hyperechoic linear structure in the central portion of the testicle, representing the testicular mediastinum. The epididymis was identified as a hypoechoic structure and isolated from measurement. Morphometric means were measured with lengths of 2.12 ± 0.07 cm (RT) and 2.14 ± 0.13 cm (LT), widths of 1.53 ± 0.13 cm (RT) and 1.51 ± 0.07 cm (LT), and heights of 1.35 ± 0.06 cm (RT) and 1.33 ± 0.05 cm (LT). The overall mean of the volumetric estimates was 3.11 ± 0.21 cm3. Regarding testicular evaluation, a thorough examination is required, since these organs are directly involved in sperm production, and consequently affect reproductive performance. Therefore, by introducing the use of ultrasonography in the testicular evaluation of the oncillas, this study represents a significant advance, due to the definition of the technique and ultrasonic parameters of physiological echotexture and echogenicity applicable to the species, similar to those previously described for the domestic cat (3). In large felids, testicular ultrasound has already proven effective for diagnosing testicular alterations in jaguars (Panthera onca), and more recently in pumas (Puma concolor) to assess and classify their reproductive capacity (4). With this perspective, the present work demonstrates the application of testicular ultrasonography in oncillas for evaluation of testicular parenchyma and biometric measurement, which can be considered an initial step in the use of this tool to assist in the conservation of the species and, potentially, of other small neotropical felids. Therefore, we contributed to the understanding of the reproductive morphophysiology of this ecologically vulnerable species, which is a fundamental advance for the implementation and optimization of reproductive biotechnologies as a conservation strategy.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

EFFECT OF THEOPHYLLINE ON THE *IN VITRO* FECUNDATION OF SHEEP OOCYTES ON THE *IN VITRO* PRODUCTION OF EMBRYOS

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Sperm capacitation and the acrosome reaction are essential stages for in vitro fertilization of oocytes (IVF) during in vitro embryo production (IVP). Consequently, substances such as theophylline are utilized to mimic these processes. Studies have demonstrated that theophylline enhances the sperm's ability to bind to bovine and porcine oocytes matured in vitro (1, 2), as well as improves the motility of sperm with morphological defects, thereby increasing fertilization rates (3). However, research on the utilization of theophylline in the IVF medium of sheep oocytes remains limited. Hence, the objective of this study was to assess the impact of theophylline in the IVF medium on the IVP of sheep embryos. Sheep oocytes were aspirated from ovaries obtained from a local slaughterhouse using a vacuum pump at a pressure of 20 mmHg and an 18G catheter. Immediately after collection, the oocytes were subjected to in vitro oocyte maturation (IVM) in drops of control medium under mineral oil and incubated for 24 hours at 38.5 °C with 5% CO2. For IVF, semen was collected using the artificial vagina method. Subsequently, sperm were selected and the final concentration was adjusted to 1 x 106 sperm/mL. The selected spermatozoa were then incubated with mature oocytes, which were randomly divided into the following groups: CON-FIV, consisting of SOF® supplemented with 500 IU/mL of penicillin, 0.5 mg/mL of streptomycin, and 1.25 µg/mL of amphotericin B, and the TEO2.5, TEO5, and TEO10 groups, using the CON-FIV medium supplemented with 2.5, 5, and 10 mM theophylline, respectively. IVF was carried out over a period of 20 hours under the same cultivation conditions as IVM. For in vitro cultivation (IVC), the presumed zygotes were denuded and incubated in IVC medium for 48 hours under the aforementioned cultivation conditions. The results were expressed as percentages and analyzed using the Chi-square test in Epi Info software (Epi Info 7.2.5, Atlanta, GA, USA, 2021) with a significance level of 5%. Analysis of cleaved structures revealed that the TEO2.5 (44.0%) group exhibited superiority over the control (27.0%), however, it didn't differ from the TEO5 (32.0%) and TEO10 (35.2%) groups. Previous studies have indicated that 2.5 mM theophylline increases the formation of structures at the pro-nuclear stage and blastocysts (2). Additionally, it was observed that the addition of 2.5 mM theophylline along with penicillin in bovine semen led to a cleavage rate of 82% (4). However, increasing theophylline concentration to 5 and 10 mM produced similar cleavage rates but lower embryonic rates, suggesting that high concentrations may have detrimental effects on embryonic development (5). Consequently, this study concludes that the addition of 2.5 mM theophylline was superior to the control, resulting in increased rates of fertilized and cleaved structures.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Ultrastructural aspects in morphological characterization of oncilla *Leopardus tigrinus* spermatozoa by scanning electron microscopy

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Among the neotropical small felids, the oncilla Leopardus tigrinus (Schreber, 1775) is a predator ecologically vulnerable, threatened due to habitat fragmentation, hunting, and illegal trade. Considering this context, studies on the understanding of reproductive morphophysiology of this wild felid are scarce but essential for adopting appropriate conservation strategies. Within this perspective, the characterization of the sperm cell is crucial for the development and standardization of assisted reproduction techniques, as well as for the creation of genetic material banks that enable the conservation and multiplication of these biomaterials. This work aimed to elucidate ultrastructural characteristics of L. tigrinus in the Caatinga biome. Procedures were approved by CEUA-UFERSA No. 20/2023 and SISBIO No. 88201-1. Four adult individuals, aging 2.25 ± 0.32 years old, with average weight of 2.52 ± 0.15 kilograms, housed in zoos in Fortaleza, CE (Latitude: 3° 43' 6" S, Longitude: 38° 32' 36" W) and Canindé, CE (Latitude: 4° 21' 29" S, Longitude: 39° 18' 7" W) were used. All animals were undergone a physical examination to ensure that they were in good health, free from diseases and suitable for the anesthetic procedure for chemical restraint. Animals were captured from their enclosures using a net and chemically restrained with a combination of dexmedetomidine and ketamine hydrochloride. Semen samples were collected by electroejaculation (1), fixed in 2.5% buffered glutaraldehyde solution (pH 7.4) and refrigerated at 4ºC until being processed for scanning electron microscopy. For processing, the samples were initially centrifugated at 800 ×g for 10 minutes for cellular pellet formation. The pellets were undergone to three washes in 0.1M phosphate buffer solution (pH 7.4), impregnates in osmium tetroxide and then followed by two washes in distilled water. The samples were dehydrated with different concentrations of alcohol (50%, 70%, 90%, and 100%). After processing, the samples were mounted on stubs and coated with gold using the cathodic sputtering technique, and then observed under a scanning electron microscope (2). Ultrastructural evaluation allowed a detailed description of the sperm morphology of oncillas, showing the head of the spermatozoon of the species in an elongated oval shape, as well as enabling the definition of structures at nanoscale, such as the spermatic neck and the equatorial and apical segments of the acrosome, which had not been identified for the species yet due to methodological limitations of previous studies. Also, we identified phylogenetic characteristics that help us with the taxonomic classification by observing sperm patterns of different species (3). Relating to the oval shape of the spermatic head of oncillas, this feature has also been observed in domestic cats, pumas (Puma concolor) and cheetah (Acinonyx jubatus), which comprise the subfamily Felinae. Otherwise, Siberian tigers (Panthera tigris altaca) and jaguars (Panthera onca), both from the subfamily Pantherinae, were characterized with a more rounded shape of the spermatic head. Regarding to the spermatic neck, it was possible to identify a narrowing between the head and the midpiece, a characteristic also observed in domestic cats previously. Thus, it is suggested that this morphological characteristic is related to phylogenetic proximity. This identification provides valuable information for prospective studies that can evaluate the integrity of these structures during the application of reproductive biotechnologies, such as sample processing, refrigeration, and freezing. This study therefore allowed us to fill in the gaps in the understanding of the reproductive physiology of L. tigrinus. By describing the sperm in detail, we have contributed to providing data that can be used in the future as a reference for the species' physiological parameters to support even more effective conservation strategies.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

EFFECT OF THE ADDITION OF SULFATED POLYSACCHARIDES EXTRACTED FROM MARINE ALGAE ON THE DNA INTEGRITY OF CRYOPRESERVED SEMEN OF *Colossoma macropomum* (CHARACIFORMES: SERRASALMIDAE)

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Colossoma macropomum is a fish species native to the Amazon of significant importance, as it is highly appreciated by aquaculture, which makes it the target of scientific studies. Semen preservation makes it possible to optimize genetic improvement programs, conserve genetic variability and reduce the negative effects of inbreeding, thus ensuring the sustainability of aquaculture (1). Thus, the objective of this project was to evaluate the effects of supplementing the cryodiluter medium with sulfated polysaccharides (PS) extracted from marine algae (Ascophyllium spp or Solieria filiformis) on the DNA integrity of post-thawing Tambaqui semen. Therefore, 24 Tambaqui males weighing an average of 5 kg were separated and induced to reproduce with intracoelomic application of Ovopel® (0.3 pellet/kg BW). After 14 hours, the animals were sedated in a Eugenol solution in a ratio of 1:10:10000 (Eugenol:alcohol:water) and had the semen collected by abdominal massage in sterile 3 mL syringes. Samples with motility above 80% were used to form pools (n = 8) and each pool was formed with semen from three animals, which were diluted in a solution containing powdered coconut water (ACP-104) and 10% dimethyl sulfoxide (DMSO) and frozen with different concentrations (0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL and 0.75 mg/mL) of sulfated polysaccharides from marine algae, totaling eight treatments. After 45 days of storage in liquid nitrogen, the samples were thawed for analysis. Soon after, DNA fragmentation analysis was carried out using the chromatin dispersion test, with 200 spermatozoa per slide being counted. Those that presented an external halo were considered to have intact DNA, while the absence of a halo indicated fragmented DNA (2). The Kruskal-Wallis test and Dunn's post-test were applied, considering significant differences when $P < 0.05$. Thus, it can be observed that in samples that used Ascophyllum spp, the best result was obtained in the 0.10 mg/mL treatment, obtaining 92.0 ± 5.4a of intact DNA, while in the samples that used S. filiformis, it was obtained if the best result in the 0.75 mg/mL treatment, obtaining 88.4 ± 5.6ab of intact DNA, these results demonstrated a greater result than the previous one obtained in the control treatment (88.9 ± 6.3ab of intact DNA), therefore, it can be observed that treatments using Ascophyllum spp have a better result in maintaining intact DNA in Tambaqui semen (3).

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Animal Reproduction

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MALE REPRODUCTIVE BIOLOGY

Female effect on English Bulldog in Uruguay

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The objective of this work was to evaluate the changes produced in the testicle and prostate due to the effect of collecting and living with a heat bitch. In the experiment, 18 male English Bulldogs were used, divided into two groups of 9 dogs: living with a female in heat (HC) and not living with any female (S). Measurement of the testicles was performed with a caliper. Total scrotal width (ATE) and testicular volume were measured. Three samples were collected per animal within a period of 18 months. Semen was collected by digital manipulation, inside a preheated graduated glass collecting beaker. In both experiments, volume, pH and macroscopic appearance of the different fractions of each ejaculate were recorded. Sperm motility and vigor were evaluated immediately upon collection. Sperm concentration was established using a Neubauer chamber and the total number of sperms per ejaculate was established. An aliquot was diluted with buffered saline formalin for examination of cell morphology. In both experiments, a portable ultrasound machine with a 10 MHz linear transducer was used to perform the ultrasound. Ultrasound gel was placed on the surface of the scrotum of each testicle. Transverse and longitudinal orientations of the transducer were applied. The digitized images were processed with Image J software. The average intensity (PI) was determined from four circular areas, taken at random in each of the images of the parenchyma of the testicle and prostate. The IR value was determined by Doppler of the testicular and prostate arteries. Values are presented as mean ± standard error of the mean. Seminal parameters, PI and IR were analyzed by ANOVA with the GLM Procedure (General Linear Models, SAS). Sperm concentration and motility were higher for the HC group ($P < 0.01$) and the percentage of sperm with abnormalities was lower in the HC group ($P < 0.01$). Testicular measurements did not present differences. Pixel intensity was lower in dogs in the HC group ($P < 0.01$). IR of the testicle and prostate did not present differences. These results allow us to infer that coexistence with females in heat affects some semen parameters, as well as the testicular parenchyma, probably due to changes in testosterone production that have not yet been evaluated.

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MALE REPRODUCTIVE BIOLOGY

Kisspeptin-10 improves testicular immune status and expression of mediators of epididymal function in hypothyroid rats

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Spermatogenesis depends on the maintenance of an immune-privileged environment in the testes. However, for the sperm to acquire its functionality, it also depends on the epididymal function, since it is in this organ that the gamete undergoes maturation to enable fertilization. Furthermore, recent studies have demonstrated that kisspeptin, a key peptide for reproduction, stimulates testicular steroidogenesis and improves sperm quality in hypothyroid rats. However, there is still no information about the effects of hypothyroidism and kisspeptin on testicular immunology and epididymal function. The objective of this study was to evaluate whether hypothyroidism alters mediators of testicular immunology and epididymal function and whether treatment with Kisspeptin-10 (Kp10) could prevent these effects. Adult male Wistar rats were initially divided into control (n = 6) and hypothyroid (n = 12) groups with induction of hypothyroidism by oral administration of propylthiouracil (PTU; 4 mg/kg/day). After two months of treatment with PTU, half of the hypothyroid animals (n = 6) started treatment with Kp10 (12 µg/Kg/day), forming the third group. At the end of the third month, the animals were euthanized to evaluate plasma testosterone levels and the testicular and/or epididymal expression of inflammatory (Il-6, IL-10, TNFα), NLRP3 inflammasome complex (Nlrp3, IL-1β, Il-18, Casp1), and epididymal function (Def28, Def49, Ptgs, Aqp1, Abp) mediators. Hypothyroidism was confirmed by reduced body mass gain and free T4 levels. Furthermore, these animals exhibited lower testosterone concentrations at the end of treatment. In the testis, hypothyroidism increased testicular immunostaining of TNFα and IL-10, as well as gene expression of Il-6 and Il-10, while reducing Casp1 mRNA expression ($P < 0.05$) but did not affect Nlrp3 and IL-1β. IL-10 immunostaining was mainly in elongated spermatids and spermatozoa, while TNFα and IL-1β were observed throughout the seminiferous epithelium and interstitial tissue, respectively. Treatment with Kp10 reduced testicular TNFα immunostaining compared to hypothyroid animals and Il-18 gene expression compared to control ($P < 0.05$). In the epididymis, hypothyroidism increased the gene expression of Il-6, Ptgs, Aqp1, Def28, and Abp, while no changes were observed in the expression of Il-10, Tnf and Def49. Treatment with Kp10 reduced the expression of Il-6, Ptgs, and Aqp1, showing mRNA levels similar to control ($P > 0.05$). The findings of this study demonstrated that hypothyroidism in rats increases the expression of inflammatory cytokines in the testis and epididymis and alters mediators of epididymal function. The use of Kp10 is capable of negatively modulating the testicular and epididymal inflammatory response caused by thyroid hypofunction, as well as also reestablishing the expression of Ptgs and Aqp1 in the epididymis.

Keywords: testicle; epididymis; kisspeptin; immunology; hypothyroidism.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Sperm morphology of cooled Colossoma Macropomum semen supplemented with L-carnitine

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The tambaqui is a neotropical fish, native to the Amazon River basin, and considered of great importance to Brazilian aquaculture (1). Technological advances in reproduction and conservation have been the focus of various Brazilian researchers, with one of the most applied techniques being seminal cooling. However, this process contributes to oxidative stress and cellular damage to gametes. An alternative to improve quality is through the addition of compounds with antioxidant potential to the medium, to reduce the action of reactive oxygen species (2). L-carnitine is a naturally produced amine with anti-apoptotic and antioxidant activity. Therefore, this study aimed to evaluate the effect of using different concentrations of the amino acid L-carnitine on the sperm morphology of cooled tambaqui semen. For this, 25 male tambaqui were hormonally induced for spermiation by the application of Ovopell® (0.3 pellet.kg-1). After 14 hours, the semen was collected by abdominal massage, previously analyzed for pool formation. A portion of the pool was diluted in different treatments with powdered coconut water (T2: ACPTM-104; dilution 1:5; semen: ACP) + 0.1 mg.mL-1 of gentamicin and supplemented with different concentrations (T3- 0.75; T4 -1.50 and T5-3.00 mM) of L-carnitine. An aliquot was also cooled without adding medium to serve as control (T1). Then, the treatments were subjected to cooling at 4ºC and maintained under these conditions for zero, six, 12, 48, and 72 hours. During each period, the samples were fixed in 4% citrate formalin for subsequent analysis of sperm morphology, in which a portion of the fixed sample was stained with Rose Bengal at a ratio of 1:10 (stain: fixed semen). Smears were prepared with 10 µL of the stained-fixed semen solution, and 100 spermatozoa per slide were evaluated using an optical microscope (400x). Sperm cells were classified according to (3). Data were subjected to statistical analysis using the software "SigmaPlot 12.0". It was observed that during hour 0, there was no statistically significant difference between the treatments used. During hours six, 24, and 72, T1 was statistically inferior to the other treatments, except for T3 at hour six, where there was no difference between them. Regarding hour 48, T2 was superior to T1, T3, and T4, but did not differ from T5; furthermore, T1 was inferior to all others. From hour 6 onwards, a reduction in T1 compared to the other treatments was observed in all hours, indicating that the presence of a supplemented diluent may improve sperm quality. Other studies involving the addition of antioxidant agents in the seminal cooling medium of fish have already shown favorable results for their use (4). Thus, we can conclude that the use of L-carnitine improves the morphological quality of post-cooling semen.

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MALE REPRODUCTIVE BIOLOGY

Evaluation of sperm DNA integrity in cryopreserved semen of *Colossoma macropomum* with different concentrations of astaxanthin

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The tambaqui (Colossoma macropomum) is a fish native to the Amazon River basin and holds significant importance for aquaculture development in Brazil, being the most commercially produced native fish in the country (2). Therefore, reproductive biotechniques such as sperm cryopreservation can enhance the breeding of this animal in captivity. However, this technique may lead to cellular stress, potentially resulting in decreased structural and functional integrity of spermatozoa. Thus, the objective of this study was to evaluate the effect of supplementing the freezing medium with different concentrations of astaxanthin on the sperm DNA integrity of cryopreserved tambaqui semen. For this purpose, 10 males of C. macropomum were induced to spermiation with Ovopel® (0.3 pellet/kg body weight). After 14 hours, the animals were sedated with a Eugenol solution at a ratio of 1:10:10,000 (Eugenol:alcohol:water), and the semen was collected by abdominal massage in a cranio-caudal direction. Samples with motility above 85% were selected to form 6 semen pools. The semen pools were diluted (1:4 - semen:diluent) and frozen in a medium containing 5% glucose, BTS (Beltsville Thawing Solution), 10% dimethyl sulfoxide (DMSO), and astaxanthin at different concentrations (0.5, 1.0, 1.5, or 2.0 µM). A solution composed of 5% glucose and 10% DMSO was used as a control. The samples were frozen and, after 45 days, thawed for DNA integrity analysis using the SCD (Sperm Chromatin Dispersion) test, based on the rate of sperm chromatin fragmentation (1,3). 200 spermatozoa per slide were analyzed, cells with an outer halo indicating intact DNA, while cells without a halo indicated sperm DNA fragmentation. Data were expressed as mean ± standard deviation, and the Shapiro-Wilk test was performed, followed by the Kruskal-Wallis test and Dunn's post-test. Differences were considered significant when $P<0.05$. Samples diluted with BTS + astaxanthin at concentrations of 1.5 µM (86.00 ± 3.84) and 2.0 µM (78.00 ± 17.47), and diluted with glucose + astaxanthin at a concentration of 1.0 µM (85.28 ± 7.03), showed higher percentages of spermatozoa with DNA integrity compared to the control and other treatments. In conclusion, it can be inferred that the use of astaxanthin in the seminal cryopreservation of C. macropomum can maintain DNA integrity, and its use is recommended at a concentration of 1.5 µM in combination with BTS.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Sperm and extracellular vesicles interactions *in vitro*: validation of the protocol of coincubation of epididymal sperm and extracellular vesicles in cattle

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The extracellular vesicles (EVs) released by the apical pole of the epididymal epithelial cells interact dynamically with sperm in the narrow epididymal duct. This interaction between the EVs and sperm in the lumen of the epididymis is a key process for sperm maturation, an essential step to sperm acquisition of fertility potential. While EVs physiologically interact with sperm at the epididymis and at several parts of the male and female reproductive tract, an in vitro protocol to mimic this mechanism will help dissect its role in sperm fertility potential acquisition. Oppositive to in vivo, the coincubation of sperm with EVs to promote in vitro interaction is usually performed in a static medium without a dynamic release of EVs and with a large distance between EVs and the target sperm. In addition, the ideal duration of coincubation is elusive with some authors using 1.5 h and others using 3 h to 6 h. Herein, we aimed to validate a protocol to provide sperm-EVs interaction in vitro in bovine. Firstly, we estimated the in vivo ratio of EVs/sperm in bovine epididymis cauda at 77.69±15.26 EVs/sperm. For that, epididymal fluid was recovered from cauda of six bulls for assessing sperm concentration by Neubauer chamber and EVs concentration by NanoSight® (NTA; Malvern Panalytical). After, cauda epididymal fluids from another five bulls were collected to form a pool of EVs (epEVs) which was evaluated regarding size and concentration by NTA and morphology by transmission electron microscopy (FEI of 200 kV model Tecnai20 emitter LAB6). Next, epEVs were labeled with PKH67 Green Fluorescent Cell Linker® (Sigma Aldrich) and incubated with post-thawed epididymal sperm from three bulls after Percoll® selection in the following treatments: 500 (0.5x), 1,000 (1x), or 2,000 (2x) epEVs/sperm (concentration) and 1.5, 3, or 6 hours (time) of incubation. At the end of each period, the solution was centrifuged (600g/5 min), the supernatant was discarded, and the pellet was resuspended in Talp-Sperm to a concentration of $5x10^6$ sperm/mL that was stained with 1 µl of Hoechst 33342 0.05mg/mL and analyzed on CytoFLEX® System flow cytometer system (Beckman Coulter) for evaluation of sperm that interacted with green-epEVs. For that, 5,000 positive events for Hoechst were considered as sperm and those with higher green fluorescence intensity were considered the percentage of sperm that interact with epEVs. The median of the fluorescence intensity per sperm was also used to evaluate the sperm-epEVs interaction. Control was performed with sperm incubated with PBS stained with PKH67 for all treatments (concentration and time). For post-thawed epididymal sperm, epididymal fluid was collected from the epididymal cauda of three bulls and cryopreserved using BoviFree® (Minitube) extender. EVs were isolated by centrifugation and ultracentrifugation at 120,000g for 70 min at 4°C twice. A pool of epididymal fluid from one bull was obtained by retrograde perfusion of both epididymal cauda after slaughter. For statistical analyses, the Proc Mixed of Statistical Analysis System was used considering two class factors (concentration and time) and the donor of sperm as a random effect. Statistical difference was considered when $p<0.05$. Regarding size and concentration, epEVs showed 114.20±3.60 nm and 3.48x109±0.18x109 particles/mL, respectively. An interaction between the concentration of EVs/sperm and time of incubation was found ($p<0.0001$) for both sperm analyzed features: percentage of sperm interacting with EVs and fluorescence intensity/sperm. For both, the Control of 0.5x, 1x, and 2x showed the same percentage and fluorescence intensity/sperm independently of the period of incubation (Percentage: 1.5h in 0.5x was 2.96±2.41d, in 1x was 2.00±1.25d, and in 2x was 2.15±1.18d; at 3h in 0.5x was 3.69±2.91d, in 1x was 2.69±1.83d, and in 2x was 2.93±1.85d; and at 6h in 0.5x was 3.09±2.60d, in 1x was 2.92±2.23d, and in 2x was 2.68±1.71d. Fluorescence intensity/sperm: 1.5h in 0.5x was 0.18±0.03d, in 1x was 0.18±0.01d, and in 2x was 0.20±0.01d; at 3h in 0.5x was 0.18±0.007d, in 1x was 0.19±0.006d, and in 2x was 0.21±0.006d; and at 6h in 0.5x was 0.17±0.003d, in 1x was 0.18±0.003d, and in 2x was 0.20±0.01d). On the other hand, the treated groups with 0.5x, 1x, and 2x displayed different percentages and fluorescence intensity/sperm in 1.5h, 3h, and 6h of incubation with higher interaction depending on time and epEVs concentration. In that regard, the percentage of sperm interaction with epEVs at 1.5h in 0.5x was 13.28±2.85c, in 1x was 28.01±3.98b, and in 2x was 51.26±6.13a; at 3h in 0.5x was 19.82±4.88c, in 1x was 42.13±3.87b, and in 2x was 72.93±1.13a; and at 6h in 0.5x was 32.09±6.54c, in 1x was 68.82±6.40b, and in 2x was 85.84±2.64a. For the green fluorescence intensity/sperm at 1.5h in 0.5x was 0.33±0.02c, in 1x was 0.53±0.03b, and in 2x was 0.83±0.08a; at 3h in 0.5x was 0.44±0.02c, in 1x was 0.71±0.05b, and in 2x 1.21±0.02a; and at 6h in 0.5x was 0.60±0.05c, in 1x was 1.14±0.13b, and in 2x was 1.74±0.17a. Thus, the interaction between epEVs and sperm relies on the time of incubation as well as the concentration of EVs/sperm. To define the more appropriate protocol, we recommend testing the sperm features and fertility potential after incubation.

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MALE REPRODUCTIVE BIOLOGY

Effects of adding creatine to post-thaw equine semen on sperm quality

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The use of biotechnologies such as semen preservation and artificial insemination are routine practices in equine reproduction. However, when equine semen is frozen, sperm quality is inferior to both fresh and cooled semen. Therefore, the addition of substances that stimulate sperm metabolism and possess antioxidant properties may be an alternative to improve the quality of frozen-thawed equine semen. Creatine is an amino acid compound that acts cyclically in the rephosphorylation of ATP through the enzymatic reaction of creatine kinase, transferring a phosphate group to free ADP molecules. In this way, it increases sperm metabolism via ATP production. However, it is not yet known if it has antioxidant effects on sperm. The aim of the present study was to evaluate whether different concentrations of creatine added to post-thaw equine semen increase motility and kinematics, and the percentage of sperm with intact and functional plasma membrane (PM). For this purpose, six semen straws from different stallions (n = 6) were thawed (37°C, 30 sec.). Subsequently, the semen was distributed into four samples according to the creatine concentration: 0- (control), 0.5-, 1-, and 2- mM of creatine. The semen samples from each treatment were evaluated in triplicate using computer-assisted sperm analysis (CASA) system for total motility (TM%) and progressive motility (PM%), curvilinear velocity (VCL µm/s), linear velocity (VSL µm/s), average path velocity (VAP µm/s), linearity (LIN%), straightness (STR%), sperm oscillation (WOB%), lateral head displacement amplitude (ALH µm/s), and beat-cross frequency (BCFHz). The percentage of sperm with functional PM was assessed using the hypo-osmotic swelling test (HOST) with distilled water according to Lagares et al. (1). Sperm with functional PM (HOST +) were those with coiled tail after adding distilled water to semen at a ratio of 1:2 (semen - distilled water), subtracted from the percentage of sperm tail defects before HOST. The percentage of sperm with intact PM was evaluated using 3% eosin vital stain with semen smears. One hundred sperm were counted, and those with unstained heads were considered to have intact membrane. Data were evaluated for normality using the Shapiro-Wilk test and for homoscedasticity using the F test. Means and standard error were analyzed using analysis of variance and compared with Tukey's test. A probability of $P<0.05$ was considered significant. No statistical difference was observed in motility, kinematics, and percentage of sperm with functional PM among the treatments ($P>0.05$). However, 2 mM creatine increased the percentage of sperm with intact PM (74.2 ± 4.7%) compared to the control (61.2 ± 2.6%, $P<0.05$). In mice, the addition of 0.5 mM creatine increased VCL and ALH, suggesting a capacitating effect on sperm (2), differently from what was observed in the present study. Creatine deficiency may influence sperm parameters such as sperm concentration and motility in roosters and humans (3). It is possible that in the present study, sperm did not have a creatine deficiency and therefore, sperm motility and kinematics were not influenced. In conclusion, 2 mM creatine added to post-thaw semen conferred protective action on the integrity of equine sperm's plasma membrane.

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MALE REPRODUCTIVE BIOLOGY

EFFECT OF L-ARGININE AS A CAPACITATING AGENT IN THE *IN VITRO* FERTILIZATION OF SHEEP OOCYTES

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In vitro fertilization (IVF) is a stage of the biotechnique of in vitro embryo production (PIV) where several substances are used in the sperm capacitation process. Heparin has been the substance of choice to carry out this process in bovine PIV(1). An alternative to heparin would be l-arginine (ARG), which is a precursor of nitric oxide and controls reactive oxygen species (ROS), enabling greater sperm capacitation (2). Therefore, the objective of the present work was to evaluate the effect of including l-arginine in the IVF medium on the in vitro production of sheep embryos. For this, oocytes were collected from ovaries from a slaughterhouse and sent for in vitro maturation (IVM) in control medium. After that, the oocytes went to IVF. The semen used was collected using the artificial vagina collection method (3), from a sheep with proven fertility. The oocytes were divided equally into four treatment groups: the CON-IVF group consisting of SOF, supplemented with 500 IU/mL penicillin, 0.5 mg/mL streptomycin, 1.25 µg/mL amphotericin B and 10% of sheep serum in estrus and the ARG10, ARG20, and ARG50 groups, containing 10, 20, and 50 mM l-arginine, respectively. After this, the presumed zygotes were subjected to in vitro culture (CIV), where the presumed zygotes were denuded by successive pipetting and subjected to in vitro culture for 48 h in control medium. Next, the rate of cleaved structures was evaluated. The results were analyzed using the Chi-square test in the Epi Info software (Epi Info 7.2.5, Atlanta, GA, USA, 2021), with a significant difference being considered when $P<0.05$. It was possible to observe that the ARG50 group presented a higher proportion of cleaved structures than the other groups ($P<0.05$). The explanation for this result is that ARG plays an important role in sperm motility, in addition to inducing capacitation and acrosome reaction (4) in addition to acting as an antioxidant or free radical in cell signaling (5), by absorbing oxygen for cellular metabolism and inactivation of superoxide anions (O2), which cause peroxidative damage to the phospholipid membrane. It is concluded that the use of L-arginine at a concentration of 50 mM improves the production of sheep embryos.

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MALE REPRODUCTIVE BIOLOGY

Analysis of the use of type III antifreeze protein associated with GAGs on the potential for membrane integrity of *Prochilodus brevis*

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Amid the need for storage and conservation of genetic material, cryopreservation constitutes an important biotechnique in the reproduction (1). Such techniques allow genetic improvements in species, such as Prochilodus brevis, which plays an important ecological role and economic potential in the brazilian's northeastern region. However, the semen freezing and thawing processes can cause cryoinjury, damaging the quality of the genetic material. Recent studies demonstrate that the use of antifreeze proteins (AFPs) in the cryopreservation solution helps maintain the lipid composition of the plasma membrane, generating greater cellular stability (2). These proteins act by lowering the freezing point, preventing the process of formation of ice crystals, preventing recrystallization and reducing freezing-induced changes in the protein pattern (3). Macromolecules with antioxidant potential, extracted from Nile tilapia skin, can also be added to the freezing medium. These macromolecules neutralize the effects of reactive oxygen species (ROS), enhancing the viability of sperm cells after thawing. Therefore, the objective of this work was to evaluate the effects of using different concentrations of type III antifreeze protein (AFP III) and glycosaminoglycans (GAGs) extracted from Nile tilapia skin in the conventional cryopreservation of P. brevis and its action on the parameter of conservation of membrane integrity. In the experiment, the pools were diluted in ACP-104 and 10% DMSO supplemented with or without different concentrations of AFP III (0.1, 1.0, and 10 µg/mL) and GAGs (0.1, 0.25, 0.5 mg/mL). The samples were packaged in French straws and frozen in a dry shipper (-196 ºC) for 15 minutes and stored in a liquid nitrogen cylinder (-196 °C). Sperm membrane integrity was assessed using the eosin-nigrosin staining method at a 1:2:2 (sperm:eosin:nigrosin) ratio. A 10-µL aliquot of this mixture was used to create a smear on a slide. Each treatment was represented by one slide, which was analyzed using a light microscope (400×, Opton Microscope; Tucsen, China). Two hundred sperm per slide were examined. Unstained sperm were considered to have intact membranes, while those stained pink/red were classified as having ruptured membranes. As for membrane integrity, the treatment involving 0.25 mg/mL and 0.5 mg/mL of GAGs, or 0,1 and 10 µg/mL AFP III (54.12±5,66% ; 69.5±5.42% ; 55.87±4.71% and 53.81 ± 2.82%, respectively) provided the highest intact membrane rates ($P < 0.05$), while control, 1.0 µg/mL of AFP III and 0.1 mg/mL of GAGs led to the lowest values in the analysis of membrane integrity (49.25±6.01% ; 49.87±6.69% ; 50.81±0.44%, respectively). The antioxidant properties of GAGs likely contribute to membrane protection, considering that cell membranes, composed of polyunsaturated fatty acids, are susceptible to ROS attack. AFPs also contribute to sperm membrane protection by interacting with membrane components, providing greater stability to the lipid bilayer and preventing the growth of deleterious hexagonal ice crystals during freezing. Thus, the inclusion of sulfated polysaccharides, such as those found in Nile tilapia skin, which minimize oxidative stress, along with AFPs, which stabilize the membrane, can collectively help maintain sperm membrane integrity.

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MALE REPRODUCTIVE BIOLOGY

Mild heat stress impairs post-thaw mitochondrial potential and overall sperm quality in dairy bulls

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Heat stress (HS) is a hazard for bull fertility and spermatogenesis. Only few studies investigated the impact of mild-HS or considered that freezing process further impairs sperm cells post-HS exposure. We hypothesized that mild-HS will impair post-thaw sperm quality and more specifically, mitochondrial potential. A total of six adult Holstein bulls were kept indoors in collection facilities without temperature control (CRV BV, Arnhem, The Netherlands). Semen was collected with artificial vagina at 4 different dates (two in winter - mean THI = 43 and two in summer - mean THI = 62; with 7-25d interval between samples/season). All samples presented high quality pre-freezing and no difference was observed between bulls. . Semen was fully extended in a phospholipid-based media (OptiXcell, IMV Technologies, France), and after an equilibration time, the straws were gradually cooled from 4°C to -140°C in a programmable automatic freezer (DigitCool, IMV Technologies, France) and then submerged and stored in liquid nitrogen until use. Samples were randomly thawed in a water bath at 40 °C for 30s and immediately analysed in a CASA system (ISAS® D4C20, Proiser, Paterna, Spain), additionally, an eosin-nigrosin smear was used for morphology assessment (n=200 cell). Sperm cells were diluted to 2.5x106 cells/ml in HEPES-TALP and stained (15 min) with two fluorescent dyes: 1 µL of SYTOXTM (dead cell staining – Thermo Fisher, Eugene, Oregon, USA) + 3 µL of MitoStatus Red (mitochondrial potential - BD Pharmingen™, San Diego, California, USA). The samples were then analysed through flow cytometry (Cytoflex - Beckman Coulter Inc., Atlanta, Georgia, USA). Overall, samples from summer presented lower quality post-thaw compared to winter samples. Regarding sperm kinetics, for winter vs summer, significant difference ($P<0.05$) was observed for total motility (73.27 ± 1.75 vs 51.75 ± 1.83%) and progressive motility (61.27 ± 2.27 vs 37.52 ± 2.33%) as well as VCL (108.55 ± 2.80 vs 99.96 ± 2.31) , VSL (51.14 ± 1.12 vs 44.17 ± 1.20) and VAP (68.58 ± 1.66 vs 58.78 ± 1.49) respectively. In addition, sperm normal morphology was higher in winter vs summer (79.58 ± 0.84 vs 71.25 ± 1.66). Lastly, when performing flow cytometry, mitochondrial potential and number of live cells were higher ($P<0.05$) in winter vs summer (64.10 ± 1.82 vs 57.32 ± 1.66 and 57.14 ± 1.48 vs 34.94. There is a clear impact of the mild-HS observed in the summer on sperm quality in dairy bulls. Interestingly, an association can be observed between sperm kinetics and mitochondrial potential. Moreover, the oxidative stress through which the sperm are subjected during cryopreservation is leading to more intense impairment of the sperm mitochondria and overall quality post-thaw, indicating that even a mild-HS exposure has important consequences for sperm quality.

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MALE REPRODUCTIVE BIOLOGY

SUPPLEMENTATION OF CRYODILUENT MEDIUM OF *PROCHILODUS BREVIS* SEMEN WITH TAURINE AND MELATONIN ON SPERM MOTILITY

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The Brazilian bocachico (Prochilodus brevis) is a rheophilic fish native to the semi-arid region of northeastern Brazil. In view of its significant economic and ecological potential, reproductive biotechniques are being applied, such as sperm cryopreservation, which consists of gamete conservation through drastic temperature reduction. Exposure to thermal shock and oxygen variations can lead to the generation of reactive oxygen species (ROS), triggering various deleterious effects on spermatozoa. In order to mitigate such damage and improve post-thaw sperm quality, agents with antioxidant potential, such as taurine and melatonin, can be added to the diluent solution. Hence, the present study aims to evaluate the effects of two substances - taurine and melatonin - at two concentrations during the freezing of P. brevis sperm, with emphasis on the post-thaw motility parameter. Twenty-four mature males of P. brevis induced with 0.5 ml/kg of synthetic hormone Ovopel® were selected. After 14 hours, the animals were sedated in Eugenol solution, and seminal collection was performed through abdominal massage. The semen was collected using 3 ml syringes and subsequently subjected to motility analysis. Those presenting values above 90% were used to make seven pools (n = 7) (3 males per pool), which were diluted in medium containing 5% Glucose, 10% DMSO, and 5% egg yolk, supplemented with Taurine (1 or 3.16 mM) or Melatonin (1.12 or 3.56 mM), totaling four treatments and one control (without addition of supplements) subjected to the cryopreservation process. For the analysis of total motility (%) and curvilinear velocity (VCL - µm/s), Sperm Class Analyzer software (SCA, Microptics - Barcelona - Spain, version 3.2) was used. Statistical analyses were conducted using R software. The Kruskal-Wallis test was used to verify significant differences between treatments. Data were subjected to Analysis of Variance (ANOVA) and expressed as mean ± standard deviation. The significance level adopted was $P < 0.05$. As a result, there was no significant difference regarding the total motility parameter between the control (34.37 ± 7.73%) and the treatments supplemented with taurine 1 mM and 3.16 mM (34.23 ± 14.49% and 36.04 ± 8.79%), respectively. The same was observed for VCL since the values of the control treatment (26.26 ± 1.79) did not differ statistically when compared to taurine concentrations at 1 mM (27.89 ± 5.93%) and 3.16 mM (28.26 ± 4.99%). When supplementing the media with melatonin, the total motility rate, when using concentrations of 1.12 mM or 3.56 mM (39.63 ± 10.04% and 48.01 ± 11.40%), and VCL at a concentration of 1.12 mM (36.04 ± 8.79%), showed no statistical differences compared to the control. However, the treatment supplemented with 3.56 mM of melatonin showed superior results (36.25 ± 2.48%) compared to the control treatment. Thus, in order to improve the quality regarding sperm kinetic parameters, supplementation of the seminal freezing medium of Prochilodus brevis with 3.56 mM melatonin is indicated. It is suggested to evaluate higher concentrations of taurine and melatonin in additional tests.

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X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Effect of the natural drugs: Doxorubicin and Withaferin A on the seminiferous tubules of mice after different exposure frequencies on the *in vitro* culture

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Chemotherapeutic agents can induce various adverse effects, primarily in the testicles, owing to their gonadotoxic effects, potentially leading to infertility. Thus, several researchers have been dedicated to searching for natural substances that can reduce testicular alterations, allowing for normal spermatogenesis [1]. Therefore, the present study investigated the effect of the drugs Doxorubicin (DOXO) and Withaferin A (WTA) at different exposure frequencies during in vitro culture (IVC) of testicular fragments on the morphological structure of seminiferous tubules. For this purpose, testicles from 8-week-old mice (n = 6) obtained from the Experimental Biology Center at the University of Fortaleza (UNIFOR) were fragmented (5 mm³) and randomly allocated to the following treatments: non-cultivated control (CTR); α-MEM+ with 1% dimethyl sulfoxide (DMSO); 0.3 µg/mL DOXO; 0.6 µM and 6 µM WTA. DOXO and WTA were added to the culture medium at two exposure frequencies: only at D0 (1X) or at each change of culture medium at D0, D2 and D4 (3X). 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 IVC of the testicular fragments was carried out in 24-well plates on a 1.5% agarose membrane at 34 °C, 6% CO2 in air for 6 days, with a total change of medium every 2 days. The treatments were evaluated for histomorphology (scores of nuclear and epithelial changes) and the diameter of the seminiferous tubules, using hematoxylin-eosin staining. For statistical analysis, the Kruskal-Wallis H test and Mann-Whitney U test were utilized, with results considered significant when $p<0.05$. Regarding nuclear alterations, DOXO (3X) demonstrated a higher ($p<0.05$) percentage of alterations compared to both DMSO and WTA 0.6 (3X). Concerning epithelial alterations, when exposed at a single dose (1X), lower percentages of alterations ($p<0.05$) were observed in DOXO compared to WTA 6, but with no differences with WTA 0.6. Additionally, DOXO (3X) showed higher nuclear and epithelial alterations ($p<0.05$) compared to DOXO (1X). Furthermore, DOXO (1X) exhibited lower ($p<0.05$) epithelial alterations compared to WTA (1X). Moreover, the higher exposure frequency (3X) to WTA resulted in greater nuclear and epithelial alterations compared to both DOXO and DMSO. Regarding the diameter of the seminiferous tubules, regardless of the exposure frequency, the tested drugs did not exhibit differences among them ($p>0.05$). Habas et al. (2018) demonstrated that a single exposure (1X) to high doses of DOXO (0.1 and 0.5 mg/ml) during IVC of mouse testicular fragments resulted in damage and alterations in germ cells. A study on cancer stem cells [3] at concentrations of 1.5 and 5 uM of WTA inhibited cell migration, noting that at high concentrations it is toxic. Therefore, our findings suggest that WTA is toxic to testicular tissue and can lead to infertility.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Case report: Adjusting semen freezing and insemination protocols to achieve pregnancies from an Arabian horse stallion with poor frozen sperm quality

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The present report is based on the case of a 13yo Arabian stallion, twice gold champion at the Arabian horse championship with 68% DNA fragmentation average in fresh semen (Tunel assay) and globally recognized for producing poor-quality frozen semen straws. According to his reproductive history, the only way to achieve pregnancies was by using fresh semen, splitting the entire ejaculate between a maximum of two mares, and inseminating them close to ovulation timing. In 2023, this stallion arrived to the United Arab Emirates for a brief rest period between international competitions. During this time, the opportunity was taken to attempt pregnancies and produce frozen semen straws for use in reproductive programs after his departure. Initially, 0.5ml straws from three imported batches used without success were evaluated (CASA AndroScope – Minitube/Germany; Equine profile), being the mean results obtained as follows: 94.3±47.5x106 sperm/ml, 21.6±8.6% progressive motility (PM), 30.5±11.8% viability (Eosine-Nigrosine test - EOS), and 16.9±2.9% membrane functionality (HOST). These parameters were lower than the cutoff values suggested for straw approval, which are a minimum of 200x106 sperm/ml, 30% of PM, and 50% for each viability and membrane functionality. Before starting the semen freezing process, four ejaculates were collected with the following purposes: to analyze the raw semen quality (1), to evaluate six cooling extenders for up to 72 hours of storage at 5°C (2), to observe the influence of semen collection interval on semen quality (3), and to determine the average daily sperm output (4). The findings were as follows: (1) 65.6±6.6% of PM, >90% good sperm morphology with greater than 50% of viability and membrane functionality in both EOS and HOST tests; (2) Only 2 cooling extenders kept PM greater than 50% after 24 hours (Beyond and MAX Semen Full); (3) A second collection with less than a 24-hour interval produced lower quality ejaculate; (4) The ejaculates produced an average of 5.066±1.375x106 total sperm. Considering these results, an additional four semen collections were conducted at 48-hour intervals to perform progressive freezing trials. These trials involved split ejaculates to be frozen with a combination of the two approved cooling extenders, using three centrifugation methods, six freezing curves, and eight freezing extenders. In total, 63 freezing protocols were tested, of which 49 achieved less than 30% post-thaw PM (37˚C/30 sec), while 14 showed greater than 30% PM. The best five protocols, which exhibited an average post-thaw PM of 56.13±2.96%, were selected. Samples processed by these five protocols also produced acceptable straws regarding viability and membrane functionality tests. However, only two were finally chosen after undergoing an additional motility evaluation (SBS test) that combined osmotic membrane stress and a short temperature-resistance test, diluting the straw content after thawing with 1.5ml of milk-based extender and incubating the sample at 37°C/30min before CASA analysis. The approved protocols led to similar semen quality parameters after thawing (mean PM: 53.6±0.9%; mean EOS: 50.9±0.7%; mean HOST: 56.1±7.6%; mean SBS: 55.6±0.3%). They all shared the same centrifugation extender (Beyond – Minitube/Germany), but two different combinations of freezing extenders and freezing curves: 1) Violet (Minitube/Germany) with a manual curve using a neopor box and liquid nitrogen vapours with 60min of equilibration at 5°C minutes, and 2) STAR (Rancho das Americas/ Brazil) with a 2 hours-long curve from 22°C to -192°C using IceCube freezer (Minitube/Germany). Once the mentioned best freezing protocols were established, they were alternatively used to freeze 16 ejaculates, according to the time and nitrogen availability (a greater N2 consumption is faced when using the IceCube freezer), without noticing quality differences and with all batches being approved. The following mean results were obtained: post-thawing PM 53.7±4.5%, straw concentration 203.9 +/- 27.1x106 sperm/ml, HOST 57.8±3.8%, EOS 56.0±5.1%, and SBS 40.9±9.2% (raw semen PM: 68.8±2.6%). To determine the minimum insemination dose for this stallion, six hCG-induced mares were subjected to deep-horn insemination post-ovulation as follows, aiming to achieve the described total millions of motile sperm per dose: 1 x 100x106 (2 straws), 1 x 200x106 (4 straws), 2 x 400x106 (8 straws), and 2 x 600x106 (12 straws). However, no pregnancies were obtained. In a second insemination trial, four mares were used, and the total motile sperm amount was increased to 800x106 and 1.200x106, using 16 and 24 straws, respectively. One mare in each group became pregnant, and the conceptus developed as expected, with embryo heartbeats noticed after 21 days of gestation. Following this result and considering

the relevance of this stallion, the insemination dose to be used was fixed at 24 straws from the produced batches. Another 7 inseminations were performed, with 2 pregnancies confirmed and two embryos collected from a donor with bilateral double ovulation and confirmed subsequently in the recipients. In conclusion, despite the high percentage of DNA fragmentation in this stallion`s raw semen, the total sperm count, PM, sperm morphology, and membrane quality were all good enough to produce high-quality frozen semen batches. Therefore, labelling the stallion as a "bad freezer" was not adequate in this case, considering its tiny PM loss after freezing compared to its raw characteristics; rather, the low-quality imported straws were the result of inappropriate semen handling. Once the most appropriate semen collection schedule and freezing protocols were determined, semen production was standardized, and the straws passed quality control. The insemination dose varies according to each stallion, and more attention needs to be paid to stallions with special issues to establish the minimum effective dose. By collecting and analysing all related data, it is possible to individually customize protocols to improve fertility rates, even for subfertile stallions, as demonstrated in this case.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

ANTIOXIDANT EFFECT OF CONDITIONED MEDIUM FROM SKELETAL MUSCLE CELLS DURING *IN VITRO* CULTURE OF TESTICULAR TISSUE

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Infertility is a pathological condition that is increasingly affecting individuals of reproductive age. This is due to various factors, including lifestyle habits such as smoking, alcohol consumption, and sedentary behavior. A reduction in fertility of up to 50% is projected among the global population if the currently established trend persists. In this context, some therapies, such as physical exercise, are being studied in an attempt to mitigate these deleterious effects. The beneficial influence of physical activity on reproduction is reported due to a variety of substances produced during an exercise session, but the mechanisms by which this occurs are not fully understood. Therefore, the aim of the present study was to evaluate the effect of conditioned medium from skeletal muscle cells after a session of physical exercise on the activity of the enzyme catalase during in vitro culture of testicular tissue. For this purpose, Wistar rats underwent aerobic training for two weeks. After the training period, the femoral muscles of the right and left legs were dissected for subsequent in vitro culture for 24 hours, with the conditioned medium collected at the end of this period. Testes were collected from healthy Wistar rats aged 8 weeks. They were then fragmented (3x3x1cm) and placed in 24-well plates, containing control medium (alpha-MEM + 1.25 mg/mL bovine serum albumin) or control medium supplemented with different proportions of conditioned medium (25, 50, 75, 100%) to a final volume of 1 mL. Culture was conducted at 37°C in a humidified atmosphere with 5% CO2 for 24 hours. At the end of the culture period, the tissue was collected for biochemical analysis, and the activity of the catalase enzyme was quantified by absorbance (244 nm) using hydrogen peroxide as substrate. As a result, it was observed that the activity of catalase remained unchanged in the cultured groups, maintaining its levels similar to the fresh control ($P>0.05$). This is a positive factor, considering that the balance of the antioxidant defense system is of fundamental importance for cell function and the maintenance of events that occur during spermatogenesis. Thus, it can be observed that the conditioned medium 75% from skeletal muscle cells did not exert cytotoxic effects on testicular cells, promoting the maintenance of redox balance.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

SPERM KINETICS OF TAMBAQUI POST-THAWING SEMEN SUPPLEMENTED WITH SULFATED POLYSACCHARIDES FROM MARINE ALGAE (*Kelps sp* or *Solieria filiformis*)

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Tambaqui (Colossoma macropomum) stands out in national aquaculture due to its tasty meat and good acceptance in the consumer market (1). These characteristics have led to a growing interest in its cultivation, consolidating it as the most commercially traded native species in Brazil (2). For the captive reproduction of this species, the conservation of its gametes can be achieved using the seminal cryopreservation technique. However, the application of this technique contributes to oxidative stress on cells. Thus, the addition of substances with antioxidant potential, such as sulfated polysaccharides (PS) from marine algae, may enhance the semen cryoprotective medium. The study aimed to evaluate the effect of different concentrations of PS on the kinetic parameters of tambaqui spermatozoa. After approval by the Ethics Committee for Animal Research at the State University of Ceará (n° 09279405/2021), 24 tambaqui males, with an average weight of 5 kg and indicative characteristics of reproductive maturity, were used. The animals received an application of Ovopel® (0.3 pellet/kg) intracoelomically. After 14 hours, they were sedated in a solution of Eugenol in the proportion of 1:10:10000 (Eugenol:alcohol:water), placed in lateral recumbency, and the semen was collected by abdominal massage using sterile 3 mL syringes. Semen samples with motility above 80%, after activation, were used to form pools (n = 8), and each pool was formed with semen from three males. The pools were diluted (1:9 semen:diluent) and frozen in a solution containing powdered coconut water (ACP-104) and 10% dimethyl sulfoxide (DMSO). The cryopreservation medium was supplemented with different concentrations (0.10 mg/mL and 0.50 mg/mL) of sulfated polysaccharides from marine algae (Kelps sp. or Solieria filiformis), totaling four treatments. An unsupplemented solution was used as a control. The samples were packaged in 0.25 mL straws, sealed with polyvinyl alcohol, and left for 10 minutes at approximately 10°C for equilibration. The straws were then placed in a dry shipper, where they remained for 30 minutes for freezing in liquid nitrogen vapor (-170°C) and then stored in liquid nitrogen (-196°C). After 45 days, the samples were thawed by immersion in a water bath at 45°C for 8 seconds. Data were expressed as mean ± standard deviation. Normality was verified using the Shapiro-Wilk test. The non-parametric Kruskal-Wallis test was used to evaluate differences between groups. Finally, Dunn's post-test was employed to make multiple comparisons between groups. Differences were considered significant when $P < 0.05$. Among the results obtained for motility (%), VCL, VSL, and VAP (µm/s), significant differences were observed between the PS treatments of both species of marine algae. However, only the concentration of 0.10 mg/ml (Kelps sp) showed better motility percentages (24.37 ± 1.77) and higher velocity parameters, VCL (20.33 ± 2.04), VSL (4.81 ± 0.91), and VAP (8.37 ± 0.52) when compared to the control and the concentration of 0.50 mg/ml (Kelps sp). Whereas in both concentrations of S. filiformis, significantly higher values were obtained than the parameters observed in the control, however, between the concentrations (0.10 mg/ml and 0.50 mg/ml), no significant differences were observed. Based on the results obtained, it is suggested that the concentrations of 0.10 mg/ml (Kelps sp), 0.10 mg/ml, and 0.50 mg/ml (S. filiformis) of supplementation are capable of maintaining sperm quality, since the use of supplementation in the medium may behave in a species-specific manner and be dependent on the characteristics of the polysaccharides and their concentration (3). However, further studies are still needed to evaluate the appropriate concentrations of supplementation for both species (Kelps sp. or S. filiformis) in order to preserve post-thaw sperm quality.

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Animal Reproduction

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MALE REPRODUCTIVE BIOLOGY

DOCKING AND MOLECULAR DYNAMICS FOR UNDERSTANDING THE EFFECT OF STYRENE ON BOS TAURUS BSP1

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Environmental pollution has direct impacts on human and animal health. Among pollutants, plastic has drawn the attention of the scientific community due to its increased global production in recent decades. Studies already show that its components pose severe health risks (1). Additionally, in the environment, plastic debris has the potential to further degrade or fragment into micro and nanoplastics (2). These have negative effects on the reproductive system of different species, which can lead to infertility. Thus, there is a perspective on understanding how pollution at the molecular level can affect fertility-related seminal proteins, such as Binder of Sperm Protein (BSP), which is closely linked to sperm capacitation. In fact, the issue is related to the omnipresence of nanoplastic, involving its size, which facilitates its entry into the animals' food chain, generating negative effects on reproduction and potentially interfering with various segments of the reproductive process, thereby affecting fertilization rates. Therefore, the aim was to evaluate the impact of Styrene (STY) on BSP1 in bulls, using computational biology approaches, with the use of molecular docking and molecular dynamics, intending to investigate the structural, functional, and interactional alterations of BSP1 induced by exposure to styrene. The ProteinPrepare server software was used, as well as MarvinSketch 18.24 and Discovery Studio 2020, for adjustments in protein and ligand (BSP and STY) protonation, respectively, at pH 7.4, and subsequently, molecular docking was performed using AutoDock Tools 1.5.6 and AutoDock Vina 1.1.2, and then the pose with the most attractive docking score was selected for the molecular dynamics (100 ns) of the BSP:STY complex using GROMACS 2021.2. The molecular docking assay showed that STY binds to BSP1, which can be visualized in its major binding at the fibronectin type II (FN2) site 2, responsible for the protein's ability to bind to cholesterol and phospholipids. Regarding the molecular dynamics assays, instabilities were observed in the BSP:STY complex, which may indicate that I) the bonds do not occur at the sites indicated by molecular docking or II) STY may cause a structural change in the protein, which may have a negative effect on cholesterol efflux in the sperm membrane, negatively affecting sperm capacitation, and consequently animal fertility. Quantum biochemistry studies should be conducted to investigate the bonds formed between STY and BSP, as well as the residues involved in the protein-ligand complex interaction. These data can contribute to future studies on minimizing the effects of pollution on fertility.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Histological comparison of gonads from male *Prochilodus brevis* during reproductive and non-reproductive season

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The Prochilodus brevis (Brazilian bochachico) is a rheophilic and endemic fish of the semi-arid region of the Northeast, also being the area of greatest commercial focus due to the gastronomic appreciation for its roe (1). However, due to predatory fishing, which mainly occurs during the pre-spawning period (2), and human interventions that prevent the species' migration during the reproductive season, which occurs between November at May, the species in question presents difficulty in surviving in the wild, requiring captive breeding and reproduction (2,3). For this reason, knowledge of the morpho-histological characteristics of the gonads is essential for understanding fish reproduction (3). Regarding the morphology of the species' testes, it is possible to characterize and classify them into four reproductive stages, which occur spontaneously in animals in the wild and in captivity. However, for gametogenic release in captivity, human intervention is necessary (1,3). The curimata's testes are paired and connected by the final portion forming a single spermatic duct. There are alterations in volume, color, and thickness in the outer region according to each phase of development and maturation (1). Outside the reproductive season, when immature, the gonad is thin and small, translucent, containing only gametogenic cells in early stages of development, and from a microscopic point of view, it is not possible to observe the seminiferous tubules. During maturation, which occurs only during the reproductive season, the seminiferous tubules are visualized, and there are cysts of different phases of the spermatogenic lineage in the lumen. When mature, the gonad's coloration is milky white, and spermatozoa accumulate in the seminiferous tubules' lumen until spermiation. In the last observed stage, called regression, the germinal epithelium is discontinuous with vacuoles and thick interstitium, and the seminiferous tubules' lumen appears with remaining spermatozoa (1,2). Due to the species' lack of sexual dimorphism, even during the reproductive season, further studies on reproduction are necessary for the improvement of P. brevis cultivation (3). In this species, males and females show expansion of the celomic cavity to accommodate the gonad size, which increases during the reproductive season (1). Thus, the objective of the study was to perform histological analysis of the gonads of male P. brevis to evaluate and describe the testicular structures in animals during reproductive and non-reproductive season. For this, 14 male of P. brevis were used, 5 during the reproductive season and 9 in non-reproductive season. The animals were euthanized with an overdose of Eugenol® and fragments of approximately 1 cm were collected for staging. The samples were fixed in 10% buffered formalin for 24 hours, then dehydrated, cleared, and embedded in paraffin for 5 µm sections on a microtome. After removing the paraffin, the slides were stained with Carazzi's hematoxylin-eosin. After histological evaluation, it was possible to confirm that the species is capable of reaching all stages of maturation even in captivity, as well as to correlate, through subjective analysis of the histological slides, that animals with a larger gonadal volume during the reproductive season had a higher presence of spermatozoa in the seminiferous tubules' lumen.

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Animal Reproduction

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MALE REPRODUCTIVE BIOLOGY

Spermatic parameters of Wistar rats submitted to testicular autohemotherapy as a non-surgical castration alternative

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The main method of sterilizing dogs and cats is still surgical castration (1). Despite this, negative factors such as the high cost, surgical and anesthetic risks, need for installations, equipment and qualified professionals, greater post-surgical care, longer recovery time, discomfort and pain (2) gradually increase the search for non-invasive methods. In this sense, alternative therapies are emerging, such as hormonal, immunological and chemical methods (3,4). However, for them to be considered as effective as surgical sterilization, they should be effective in the most of animals treated, interrupting gametogenesis irreversibly, in addition to being safe and accessible (3); a fact that limits most of them. Thus, the aim of this study was to evaluate the capacity of testicular autohemotherapy as a possibility of non-surgical castration. For this purpose, a total of 21 sexually mature male Wistar rats (120 days old) were randomly distributed into three experimental groups: G1=control; G2=testicular autohemotherapy with 0.7 ml of blood/testis; and G3=testicular piercing with a needle without applying any content. At the end of 60 days, all animals were euthanized, and the spermatozoa were recovered from the epididymis tail using the flotation technique in Tris-buffer solution (5). After obtaining them, the gametes were analyzed for motility under an optical microscope (10X), as well as sperm concentration using the Neubauer chamber technique (40X) (6). The data obtained were transformed by arc sine, when percentages, and analyzed by the one-way ANOVA test, followed by the T-Test and expressed as means and standard deviations (mean±SD), considering a significance level of 5%. After testicular autohemotherapy treatment, no significant differences were observed (P>0.05) between the experimental groups for motility (G1=48.51±23.94; G2=30.00±16.58; G3=54.29±15.92) and sperm concentration (G1=22.97±8.71; G2=21.84±2.96; G3=15.89±3.15). Sperm concentration is an indicator of the testicles' spermatogenic capacity (7) and, together with sperm motility, is crucial for conception (8,9). Therefore, it is concluded that autohemotherapy does not represent, in the way it was used, an effective castratition technique, since it is unable to compromise the testicular and spermatic functions.

Keywords: administration of autologous blood; spermatogenesis; sterilization; male gonads; sperm motility; sperm concentration.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

SPERM MEMBRANE INTEGRITY OF *Colossoma macropomum* AFTER SEMINAL COOLING IN CRYODILUENT SUPPLEMENTED WITH MELATONIN

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The tambaqui (Colossoma macropomum) is an Amazonian rheophilic fish of great importance for aquaculture, as it is the most commercially produced native species in the country (1). The application of biotechnologies such as sperm cryopreservation aims to enhance its reproduction in captivity. Cooling to 4°C allows the storage of semen samples for hours or days, however, sperm cells become susceptible to oxidative stress caused by excessive action of reactive oxygen species (ROS), leading to various cellular damages, such as plasma membrane rupture of spermatozoa (2). Therefore, it is necessary to supplement the cryodiluent medium with substances with potential antioxidant action, such as melatonin. This study aimed to evaluate the effect of adding different concentrations of melatonin to the tambaqui semen cryodiluent medium on sperm membrane integrity parameter at different cooling times. For this purpose, 20 males were hormonally induced for spermiation by applying the synthetic hormone Ovopel® at a dosage of 0.3 pellet/kg-1 of body weight intracoelomically. After 14 hours, semen was collected through abdominal massage. The samples were previously analyzed for motility and then cooled and distributed into the following groups: in natura semen (control 1), semen + powdered coconut water (ACP-104®) (control 2), and the remaining samples diluted in ACP-104® supplemented with melatonin at respective concentrations: 0.75 mM, 1.5 mM, and 3.0 mM, totaling 5 treatments. The samples were analyzed for sperm membrane integrity at zero time (Hour 0), 6 hours (Hour 6), 24 hours (Hour 24), 48 hours (Hour 48), and 72 hours (Hour 72) of cooling. The eosin-nigrosin staining method was performed, in which 10 µL of a mixture of semen, eosin, and nigrosin (1:2:2) were used to prepare slides by the smear method. With the aid of a light microscope (400x magnification), one slide from each sample was analyzed, where 200 spermatozoa per slide were counted and considered to have intact membrane when they were colorless or with a ruptured membrane when stained pink or red (3). For statistical analysis, SigmaPlot 12.0 software was used. ANOVA followed by Tukey's test for mean comparison was conducted, with a significance level of $P<0.05$ adopted. Data were expressed as mean ± standard deviation and presented as percentages. The results obtained showed that there was no significant difference between the control groups and melatonin treatments during Hour 0 (97.28 ± 0.98, 98.00 ± 1.09, 96.33 ± 1.60, 97.42 ± 1.32, and 97.42 ± 1.43) and Hour 6 (94.92 ± 1.91, 97.75 ± 0.82, 96.58 ± 1.49, 96.75 ± 1.89, and 95.50 ± 1.04). During Hour 24, control 1 (in natura semen) differed significantly ($P<0.05$) (77.58 ± 1.93) from the other treatments (88.58 ± 2.99, 91.25 ± 1.13, 90.66 ± 4.72, and 91.48 ± 2.58). At Hour 48, control 1 significantly differed (65.33 ± 6.24; $P<0.05$) from control 2, melatonin 0.75 mM, and 1.5 mM (78.00 ± 8.74, 75.31 ± 6.31, and 78.25 ± 6.81), however, there was no difference when compared to melatonin at a concentration of 3.0 mM (71.75 ± 5.24). At hour 72, control 1 was found to be inferior to the others (53.17 ± 8.33; $P<0.05$), and when comparing the other treatments among themselves, it was observed that there was no significant difference between control 2 (67.52 ± 4.87), melatonin 1.5 mM (67.83 ± 3.28), and melatonin 3.0 mM (66.42 ± 6.94). However, these were inferior when compared to the treatment with melatonin 0.75 mM (79.75 ± 3.42) ($P<0.05$). Thus, it can be concluded that the concentrations of melatonin used in the present study, as well as the medium ACP-104®, maintain quality regarding the sperm integrity parameter for up to 48 hours of seminal cooling. For a period of 72 hours, the use of the concentration of 0.75 mM of melatonin is recommended, as it stood out among the others during the longest seminal cooling period of C. macropomum.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Proteomic analysis of seminal plasma from fertile men and men with varicocele in Northeast, Fortaleza, Brazil

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According to the World Health Organization (WHO) and the International Committee for Monitoring Assisted Reproductive Technologies (ICMART), infertility can be conceptualized as the inability of a couple to conceive over 12 months or more with unprotected sexual intercourse without conception or full-term pregnancy, as well as couples who conceive but experience miscarriages, and those seeking medical treatment. Infertility affects 15 to 20% of the population, with 50% of these cases related to male factors, thus highlighting the significant role of male health in human reproduction. Elucidating the causes and factors leading to male infertility is important in understanding the increasing prevalence of this condition. Therefore, the aim of this study was to characterize the proteome of seminal plasma from men diagnosed with varicocele and with normal semen parameters attending an assisted reproduction clinic. Nineteen men participated in the study, divided into 4 groups: normozoospermic (5), varicocele with asthenozoospermia (4), varicocele with teratozoospermia (4), and varicocele with oligoteratozoospermia (6). A total of 724 proteins were identified, with ANX A3, PRDX1, and CRAC1 highlighted as the most abundant in the seminal plasma of normozoospermic men. In the varicocele group with asthenozoospermia, 886 proteins were identified, with IGLC3, IGLC1, and H2BC1 confirmed as the most abundant. In the varicocele group with oligoteratozoospermia, 907 proteins were identified, with TUBB, MSLN, and HEX confirmed as the most abundant. In the varicocele group with teratozoospermia, 1728 proteins were identified, with HSPA1, KIF5C, and CLTCL1 confirmed as the most abundant. This study provides the first characterization of the seminal plasma proteome in men with varicocele, identifying proteins and pathways associated with spermatogenesis, energy metabolism, fertilization, reproductive tract permeability, and immunofertility. Furthermore, miRNAs indicated functions associated with various biological processes such as embryonic development, cell differentiation, cell proliferation, programmed cell death (apoptosis), and response to cellular stresses. This study offered a detailed description of the seminal plasma proteome, serving as a descriptive study focusing on semen parameters of individuals with normal semen parameters and varicocele.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

IDENTIFICATION OF KALLIKREIN-1E2 IN CRIOLLO STALLION SEMINAL PLASMA

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Seminal plasma (SP), the product of testes, epididymis, and accessory sex glands, is a fluid released during ejaculation and represents up to 98% of the voluminous stallion ejaculate. SP plays an essential role in reproduction as a transit medium and a source of energy, antioxidants, enzymes, and minerals. The present study aimed to investigate proteomics of the seminal plasma of stallions. Twenty four Criollo stallions with a known reproductive history and per-cycle concept rate data calculated from at least 30 inseminations were used. One ejaculate was collected from each stallion during the breeding season. Pregnancy rates ranged at day 16 after artificial insemination from 20.2% to 95.6%. The animals were divided into two groups: High Pregnancy (HP), with a pregnancy rate per cycle ≥60%, and Low Pregnancy (LP), with a pregnancy rate of ≤41%. Analysis of sperm concentration, kinetics, morphology, and plasma membrane integrity and functionality were performed. The sample was centrifuged at 400 x g for 10min. SP plasma was then transferred to a 2 mL microcentrifuge tube and centrifuged again (10.000 x g, 60min, 4°C). Proteins were separated using 2D SDS PAGE gel electrophoresis in duplicate. Spots in at least 80% of the gels in one of the groups, with significant abundance ($P<0.05$) and at least a 1.5-fold magnitude difference between groups, were selected and identified. A total of 716 spots were detected, with molecular weight ranging from 12 to 225 kDa. Five spots were analyzed in the LC-MS/MS mass spectrometer, of which five had their proteins identified by the MASCOT application and validated by SCAFFOLD. For non-parametric data the test used was the Kruskal Wallis and parametric data of was analyzed by Anova and t-test. The five spots were identified with Kallikrein-1E2 (KLK) that showed higher abundance in HP than LP ($P<0.05$). The KLK1E2 proteins is a member of the mammalian serine protease gene and is regulated by androgens. KLK1E2 is one of the major proteins found in equine seminal plasma. Protein KLK1E2 is correlated with sperm motility parameters (total motility and progressive motility). KLK1E2 proteoforms in seminal plasma are involved in climate conditioning of reproduction, which corroborate with our study, as all stallions were collected during the breeding season (spring–summer). Seminal plasma, the fluid fraction of the semen, contains fundamental components for spermatic capacitation and fertility. The presence and amount of proteic and non-proteic biomolecules may indicate the quality of the semen. In conclusion, the proteins are essential to the biological processes related to stallion fertility, providing insight into the proteins within seminal plasma that could serve as biomarkers for fertility in stallions.

Keywords: Seminal plasma, Stallion fertility and Proteomics.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Conditioning for collecting semen from captive Antillean manatees (*Trichechus manatus manatus*) in Northeast Brazil

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The Antillean manatee (Trichechus manatus manatus) belongs to the order Sirenia and is one of the most endangered aquatic mammal species in Brazil, where we two living species of this order are found, with an area of sympatry with confirmed occurrence of hybridization. Despite advancements, reproductive aspects remain poorly understood, including sperm evaluation. Thus, the present study aimed to carry out conditioning for the collection of semen from two captive manatees held in CMA/ICMBio facilities in Pernambuco and Alagoas states, Northeast Brazil, followed by subsequent analysis for morphological characterization of spermatozoa. Ethograms were conducted from January to March 2024 to establish the semen collection method. Behavioral conditioning and human touch were employed to induce the manatees to expose their penis, followed by manual movements to stimulate ejaculation. Subsequently, a smear was prepared from the collected sample and stained with gentian violet to verify the presence of spermatozoa. Seminal evaluation was performed using a Nikon Eclipse E200 microscope with 4x/0.10 and 10x/0.25 magnification objectives. Both animals demonstrated positive behavior during conditioning. However, only one specimen, housed in a natural environment, successfully yielded semen, with the presence of spermatozoa and the other containing only seminal fluid. This study marks the first report of manatee semen collection in Brazil and the second globally (Front. Vet. Sci., v. 7:569993, 2020). Although preliminary, our findings demonstrated the feasibility of the conducted semen collection and conditioning. This experiment lays groundwork for future studies, not only concerning the studied species, but especially the hybrids found in Brazil, as well as the reproductive impact of natural hybridization. However, it is recommended that these studies should be limited to specimens unsuitable for release, as conditioning may interfere with the successful adaptation to natural environments upon release.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Ultrasound evaluation of testicles and seminal glands in Rusa deer (*Rusa timorensis*) raised in Northeastern Brazil

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The Rusa deer (Rusa timorensis) is one of the nine species of deer reported in Indonesia, and despite being quite common in captivity in several countries around the world, it is subject to the process of inbreeding degradation, as its native population is irregularly distributed in a small geographic area, being classified as vulnerable on the Red List of the International Union for Conservation of Nature (IUCN). In order to preserve endangered species, it is necessary to optimize reproductive conditions and concerning the male, the use of modern tools, such as ultrasonography, is important to select the best individuals in order to enable the conservation programs. Additionally, raise this species in regions different from its natural habitat should also contribute to its preservation. Thus, the objective of this study was to use ultrasonography to verify characteristics of testicles and seminal glands in Rusa deer raised in a semi-arid region of Northeastern Brazil. Three males (3-4 years old and 100.6 ± 18.8 kg) from a commercial breeding farm (Caucaia, Brazil, 3°44'10" S and 38°39'11" W) were evaluated. After chemical restraint, data on scrotum circumference (SC), testicles (width - Wi, length - Lh and thickness - Th) and testicular volume (4/3 × ¶ × (TL/2)2 × (h/2)) were recorded. The observations were made individually from each testicle (right - RT and left - LT) using a measuring tape and caliper. An ultrasound scanner (ExaPad Mini, IMV, L'Aigle, France) was used to observe echotexture from testicles through images obtained from a B-mode linear transducer. Thus, six square areas of 9.0 mm2 in the testicular longitudinal section and four in the transverse section were analyzed. The same both equipment and mode were used for visualization of right (RSG) and left (LSG) seminal glands using a transrectal transducer. The testicular images obtained were subjected to analysis using the ImageJ v. 1.46 software (National Institutes of Health, Bethesda, USA). The intensity pixel average (Numerical Pixel Values - NPV) was defined as grayscale values of individual image elements ranging from 0 (absolute black) to 255 (absolute white). On palpation, the three animals had testicles of normal consistency. The means (± standard deviation) of testicular measurements were 19.7 ± 1.9 cm for SC, 5.6 ± 1.3 cm for RTLh, 5.6 ± 1.5 cm for LTLh, 3.3 ± 0.7 for RTWi, 3.1 ± 0.4 cm for LTWi, 3.1 ± 0.7 cm for RTTh, 3.1 ± 0.3 cm for LTTh. The calculated volumes were 33.8 ± 20.6 cm3 for right testicle and 29.1 ± 14.3 cm3 for left testicle. Concerning the seminal glands, the following values were verified: 31.6 + 5.8 mm (RSG height), 30.8 + 6.1 mm (LSG height), 11.0 + 2.1 mm (RVG width) and 10.6 + 2.0 mm (LVG width). No bulbourethral glands were visualized, as expected for Rusa deer. The NPV of the testicular images in transverse cross-section were 65.0 (right) and 63.8 (left). The testicular NVP measurements in longitudinal sections were 54.5 (right) and 83.4 (right). The collected data for testicles and seminal glands of Rusa deer raised in northeastern Brazil were similar to those from native animals in Indonesia. In conclusion, the present study contributes initial information on the reproductive physiology of Rusa timorensis raised outside their original geographic region. Furthermore, this knowledge will allow the application of reproductive biotechniques to preserve this species.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Alternative use of cell labelling fluorescence non-cytotoxic

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Regenerative medicine is currently an alternative therapy for everyday complications to promote the regeneration of injured tissues using stem cells. The amniotic mesenchymal stem cells (AmMSCs) have advantages due to their attainment of biological disposal after pregnancy and their efficiency in the immunomodulatory process, an angiogenesis process with a low level of rejection due to the phenotype MHC I+ and MHC II-. These cells share the characteristics of embryonic and adult stem cells and are characterized by a lack of tumorigenic activity when injected into immunocompromised animals. However, there are no studies demonstrating the pathway of MSCs to their target organ. In this study, we aimed to evaluate the fluorophore positivity, fluorescence intensity, and longevity of canine AmMSCs. In the methodology, canine AmMSCs from the GDTI/USP (Innovative Therapies Development Group, University of São Paulo) biobank were subjected to three labeling conditions, Ctrace (CellTrace CFSE Cell Proliferation kit), Ctracker (CellTracker Green CMFDA), and also the usual methodology of genomic insertion of the gene encoding the green fluorescent protein (GFP). Analyses of fluorescence by photomacrography using ImageJ software, assessed by pixel intensity, and flow cytometry to evaluate fluorescence levels were performed. Our results showed fluorescence in all groups. However, CTrace and CTracker fluorescence was detected 4 hours after transduction and GFP was visualized within 48 hours. The photomacrograph results show the fluorescence similarity of CTrace and Ctracker compared to GFP. Flow cytometry analysis at D7 showed positive cells in all groups. These are reduced by the use of EDTA Tryple. However, the effect of labeling CTrace remained fluorescent until D32, similar to GFP. We conclude, with potential cell tracking in vitro analysis, the Ctrace, an alternative to GFP in canine amniotic stem cells, to the use of tracking fluorescent green in vivo. This tracking cell efficiency will be applied in experimental models of clinical and reproductive, providing a visualization of this cell path to future research on cell immunomodulation. Fluorescent dyes for live cells, or cell tracking dyes, play an important role in cell biology research, being used for traceability under some objective. In the present study, three different protocols for fate mapping were tested to analyze fluorophore positivity, fluorescence intensity, and longevity and to validate their use for the long-term screening of mesenchymal stem cells.

Keywords: Stem Cells; tracking; fluorescence.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Morphological and morphometric analysis of yellow-spotted river turtle spermatozoa (*Podocnemis unifilis*)

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Podocnemis unifilis is a chelonian species from the family Podocnemididae, known as the yellow-spotted river turtle or tracajá, measuring up to 50cm of carapace length. Although widely distributed in the Amazon basin, little to known about its morphological characteristics, including the reproductive aspects. Therefore, this study aimed to contribute to the reproductive biology knowledge of P. unifilis showing some morphologic and morphometrics characteristics of its spermatozoa. A spermatozoa sample was collected from the cauda epididymis from a recently died adult male P. unifilis kept in captivity at CEQUA. A slide with spermatozoa smear was stained with Panótico Rápido and two hundred spermatozoa were evaluated and measured using a light microscope and the Image J software. We observed that P. unifilis spermatozoa show a vermiform-shaped head, being elongated and filiform, and a long tail. The midpiece was not well distinguished from the head. The mean dimensions of the spermatozoa were: head+midpiece length: 14.92±1.22µm; head+midpiece thickness: 2.05±0.40µm; flagellum length: 52.20±6.03µm; and total spermatozoon length: 68.44±7.10µm. P. unifilis spermatozoa was larger than other chelonian species such as Chrysemis picta (~50-55µm) (1) and Mauremys caspica (~50µm) (2), but near to reported to Pelodiscus sinensis (~70µm) (3). The midpiece has been reported as thicker than the head in some chelonian species; however, in P. sinensis the midpiece and the head show similar thickness (3) corroborating our observations. This is the first description of the P. unifilis spermatozoa. Further studies using different stains and ultrastructural studies are necessary to better understand the similarities and differences of the spermatozoa among the chelonian species.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Platelet-rich plasma during fertilization improves the quality of bovine embryos produced *in vitro*

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The platelet-rich plasma (PRP) has been widely used in reproduction, and its beneficial effectsare explained by the content of its granules, which are rich in growth factors, cytokines, serotonin, and adhesion molecules. The serotonin released by PRP granules influences the synthesis of progesterone by granulosa cells, while among the growth factors present in PRP, insulin-like growth factor (IGF-1) stands out, known for its positive effects on spermatozoa. Therefore, the aim of this study was to evaluate the effect of adding PRP to the in vitro fertilization medium of bovine oocytes. For this purpose, 506 cumulus-oocyte complexes (COCs) were matured in vitro, and after 20 hours, the COCs were transferred to the fertilization (IVF) plate containing serum-free TALP FERT medium, being distributed into the following experimental groups: Control Group (CG - IVF medium without PRP); 2.5% Group (2.5% G - IVF medium with addition of 2.5% PRP); 5% Group (5% G - IVF medium with addition of 5% PRP); and 10% Group (10% G - IVF medium with addition of 10% PRP). Approximately 24 hours after fertilization, the probable zygotes were transferred to droplets containing synthetic oviductal fluid (SOF) and 10% fetal bovine serum (FBS). Cleavage and blastocyst formation rates were assessed on the second and seventh days of embryonic development, respectively. Embryo gene expression was evaluated on Day 8 by RT-PCR using the 2ΔΔCT method, and the endogenous genes used were the mean relative expression of ACTB and GAPDH. All statistical analyses were performed using theSigmaPlot® software (version 12.0). Analysis of variance was performed for comparison of means, using Tukey's post-test when appropriate, with a significance level of 5% ($p > 0.05$).No significant differences were found between the Control Group and the 5% Group in the cleavage rate on the second day of culture and blastocyst formation on the seventh day of culture. However, a significant difference ($p < 0.05$) was detected in Group 10%, which exhibited a lower rate of cleavage and blastocysts (GC: 91.9 ± 3,9A / 44.6 ± 9.0a; G2.5%: 87.9 ± 6.4 A /39.0 ± 14.1 A; G5%: 85.8% ± 7.4 A / 35.1% ± 11.4 a; G10%: 61.1 ± 9.9 B / 17.6 ± 4.9 b). Regarding gene expression, we chose to evaluate the 2.5% and 5% groups due to their better embryonic development rates. We identified that the expression of OCT4 ($p = 0.016$) and Interferon tau ($p < 0.001$), important markers of embryonic quality, were more expressed in the group containing 5% PRP. Additionally, we observed that the expression of HSP-70 was lower in the control group compared to the 2.5% PRP and 5% PRP groups, indicating that embryos resulting from in vitro fertilization with PRP were more resistant to stressful conditions. Regarding the expression of SOD ($p < 0.001$), an important antioxidant enzyme in the embryo's redox system, the group with 2.5% PRP had lower expression. Furthermore, in embryos from the 5% Group, there was a decrease in GLUT-1 expression ($p < 0.001$), a glucose transporter, indicating a lower carbohydrate consumption in this group. Studies have shown that PRP is rich in growth factors such as FGF, TGFβ, PDGF, IGF, and EGF, as well as binding factors such as fibrinogen and serotonin, which also improve sperm motility, viability, and mitochondrial and plasma membrane integrity after cryopreservation. Therefore, we can conclude that the addition of PRP to the IVF medium at a concentration of 5% improved embryonic quality.

Keywords: Spermatozoa; Embryo; In vitro fertilization; Platelet Rich Plasm.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Influence of testicular volume on seminal parameters of alpacas (*Vicugna pacos*)

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The alpaca (Vicugna pacos) is one of the species of South American camelids (SACs) which, together with the vicuña (Vicugna vicugna), is characterized by having fibers that are highly valued and traded globally in the textile industry. To maintain commercial production, the male plays an important role in the reproductive process and consequently in genetic improvement. Thus, there are criteria for selecting breeding males that are generally based on productive performance (body weight, age) and reproductive characteristics (scrotal size, scrotal circumference, health of reproductive organs). Therefore, the objective of this study was to evaluate the influence of testicular volume on alpaca seminal parameters. To this end, 9 breeders over 4 years old from the National Institute of Agrarian Innovation (INIA), Peru, located at 2.735 meters above sea level, were used, healthy, fed with natural pastures and supplemented with oats. After testicular biometry, carried out with the aid of a caliper, the breeders were divided into two groups according to testicular volume (VT): G1 (VT: 63-70 cm3), G2 (VT: 83-94 cm3). The semen collected using an artificial vagina incorporated into a mannequin was classified according to volume (mL), filament formation (cm), foam (cm). As well as motility (%) and concentration (sperm/mL) and viability (%). The data obtained were analyzed by the non-parametric Mann-Whitney and chi-square tests, using the R 3.4.3 software, considering $P<0.05$ as the level of significance. The results observed in animals G1 and G2 were respectively: volume of 1.37 ± 0.83 vs 2.73 ± 2.37 ($P<0.05$); consistency 1.71 ± 1.41 vs 1.94 ± 1.18 ($P>0.05$) and foam 1.72 ± 1.99 vs 2.29 ± 2.13 ($P>0.05$). Motility was 68.73 ± 15.62 vs 54.60 ± 17.97 ($P<0.05$); concentration of 124.92 ± 60.27 vs 163.76 ± 93.08 x 106 ($P<0.05$) and viability of 62.95 ± 22.08 vs 76.67 ± 15.89 ($P<0.05$). Testicular volume is a good indicator of testicular development and for many authors it is an adequate measure to represent sperm production and capacity (1)(2). Thus, it was observed that male alpacas that presented the largest testicular volume, had a greater ejaculate volume, concentration and sperm viability. From these results it can be concluded that in alpacas, testicular volume can predict their reproductive capacity, being a parameter to be taken into consideration when selecting breeding stock by breeders.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

UHT milk: a practical and cheap alternative for preserving the kinematics parameters of goat sperm subjected to refrigeration

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Skim milk powder is a base for the formulation of the most widely used goat semen extender (1). Currently, with the near-complete disappearance of instant milk without the addition of calcium, an ion related for sperm capacitation (2), this has become an obstacle into the goat semen biotechnology industry. Based on the above, the aim was to investigate the cryoprotective potential of UHT milk in preserving the kinematics of goat spermatozoa subjected to refrigeration. For this purpose, three seminal pools, coming from three goat breeders, were fractionated and diluted, in the absence of seminal plasma, in Tris-egg yolk extender [G1: 67.2mL of Tris buffer solution, 20.0mL of egg yolk (20%) and 12.8mL of milliQ water] and in UHT skimmed milk from a Brazilian industry (G2), without any modification, to a concentration of 80 x 106 sperm/mL. Then, the semen samples were refrigerated (5 °C) and evaluated at 0, 24, and 48 hours after reaching 5 °C for sperm kinetics, in a computerized analysis system (CASA) (3), considering the parameters of total motility (%), progressive motility (%), linearity (%), and straightness (%). The data obtained were expressed as means and standard deviations (mean±SD) and analyzed, after arcsine transformation, by the one-way ANOVA test, followed by the Tukey-Kramer multiple comparison test, considering a significance level of 5%. Based on the findings, no significant differences (P>0.05) were observed between G1 and G2 for the maintenance of total motility (0h: G1=91.50±3.78; G2=88.40±5.98; 24h: G1=89.63±7.94; G2=82.17±9.18; 48h: G1=76.97±27.79; G2=82.67±11.22), progressive motility (0h: G1=32.20±9.71; G2=34.90±2.34; 24h: G1=36.97±10.13; G2=34.13±19.74; 48h: G1=31.70±18.20; G2=28.27±8.81), linearity (0h: G1=46.73±9.87; G2=54.93±2.74; 24h: G1=52.53±8.61; G2=56.67±8.07; 48h: G1=53.20±4.82; G2=47.47±6.79) and straightness (0h: G1=71.67±7.39; G2=75.50±0.98; 24h: G1=75.70±7.32; G2=78.83±3.76; 48h: G1=73.53±2.78; G2=74.50±8.45). Milk is a medium rich in nutrients, such as sugars (4), and substances with cryoprotective potential, especially the casein (5), a fact that justifies the evidence about its high capacity to maintain goat sperm throughout the refrigeration (6). It is concluded that UHT skimmed milk is an accessible, practical and cheap alternative as a caprine cryoprotective extender, once that preserves the kinematics of refrigerated spermatozoa.

Keywords: goat; semen biotechnology; cryopreservation; seminal extender; functional integrity; spermatozoa progressiveness.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Plasma membrane and acrosome integrity of goat sperm refrigerated in UHT skimmed milk

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The goat farming industry is a cultural and economic highlight in the Brazilian Northeast (1). In order, to meet the growing consumer market, the use of reproductive biotechnologies has emerged, with a focus on semen cryopreservation in association with artificial insemination (2). However, in recent years, it has been difficult to obtain instant powdered milk without added calcium (2), the main base for the formulation of goat seminal extender, without compromising the structural and functional integrity of the gamete (3). The aim of this study was to investigate whether UHT (ultra-high temperature) skimmed milk has the ability to preserve the plasma membranes and acrosomal integrity of sperm. To achieve this, ejaculates from three sexually mature goat breeders were collected and formed three pools. These were previously evaluated and, when approved, intended for fractionation and dilution, in the absence of seminal plasma, in Tris-egg yolk or in skimmed UHT milk from Brazilian industry, without modifications, to a concentration of 80 x 106 sperm/mL. To remove the seminal plasma the semen pools were diluted (1:9) in Tris-buffer solution and centrifuged twice (3000 rpm/10 min). Subsequently, the samples were refrigerated and evaluated at zero, 24, and 48 hours after reaching 5 °C for plasma membrane integrity, through double staining with the fluorochromes carboxyfluorescein diacetate and propidium iodide, and acrosomal integrity, by FITC-PNA (fluorescein isothiocyanate conjugated to peanut agglutinin) technique (4). Data were expressed as means and standard deviations (mean±SD), and analyzed, after arcsine transformation, by one-way ANOVA test, followed by Tukey-Kramer, considering a significance level of 5%. According to the results, no significant difference (P>0.05) was observed for plasma membrane integrity between samples refrigerated with Tris-egg yolk (0h: 60.33±13.04; 24h: 60.67±16.10; 48h: 52.17±17.03) and UHT skim milk (0h: 61.0±6.50; 24h: 50.67±9.88; 48h: 54.17±17.74). This fact was repeated for acrosome integrity at zero and 24 hours of refrigeration (P>0.05. 0h: Tris-egg=74.17±3.25; milk=61.33±4.37; 24h: Tris-egg=63.83±9.52; milk=56.67±4.25). However, at the end of 48 hours, this parameter was better preserved (P<0.05) by Tris-egg yolk (53.67±14.47) than by milk (27.00±6.76). The cryoprotective potential of UHT milk can be attributed to its high concentration of casein, that when binding to seminal plasma proteins prevents them from causing damage to sperm cell membranes during the cryopreservation (4). Although, the sugars present in milk, such as lactose, act as energy sources by providing the carbohydrates necessary for sperm metabolism, which guarantees that they maintain their metabolic activity (6). Lactose also helps to maintain cellular osmotic balance and membrane stability, reducing the adverse effects of the low temperature stress (6). Based on the results discussed above, it can be concluded that UHT skimmed milk is a real alternative to conventional diluents to preserving the structural and, consequently, functional integrity of caprine sperm, providing convenience and economy.

Keywords: caprine; semen extender; sperm integrity; semen refrigeration.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Cryopreservation of testicular fragments obtained from goat testicles stored for different periods

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Considering the importance of preserving the reproductive potential of male goats with high genetic value, and the fact that they may die prematurely due to certain factors, strategies such as the cryopreservation of testicular tissue are of most importance in safeguarding genetic material (1). However, the success of this biotechnology depends on various aspects, among which is the storage time before the testicular fragments can be cryopreserved. Therefore, the present study aims to compare the storage of prepubertal goat testicles for 2 hours and 6 hours before cryopreservation. For this purpose, 6 pairs of testicles from non-breed specific goats aged 2-6 months were used. The testicles were obtained by surgical castration and stored in saline solution for 2 and 6 hours at 4 °C. After these periods, the testicles were dissected and cut into small fragments of 5 mm^3, which were distributed into the following groups: Fresh refrigerated for 2 hours (F-2h), fresh refrigerated for 6 hours (F-6h), freezing after refrigeration for 2 hours (C-2h), and freezing after refrigeration for 6 hours (C-6h). All fragments were subjected to histology (HE), immunohistochemistry (PCNA), and gene expression (Oct4 and C-kit). For statistical analyses, the Mann-Whitney U test was used to compare the treatments (control vs. freezing), and to compare the storage times (2 vs. 6 hours), the results were significant when $p<0.05$. The results show that epithelial alterations were greater ($p<0.05$) in the freezing (C-2h and C-6h) compared to their respective fresh (F-2h and F-6h); however, nuclear alterations were greater ($p<0.05$) only in C-2h, compared to F-2h. In the present study, refrigerated storage of testicular tissue prevented alterations or protected membrane integrity for up to 6 hours, which was in agreement with the previous study of felines (2). Additionally, C-2h testicles showed less ($p<0.05$) immunolocalization of PCNA, a cell proliferation marker, compared to F-2h, something that did not occur with the C-6h testicles that maintained PCNA marking. Thus, as seen in other studies, freezing-thawing processes directly affect cell proliferation, causing damage to the seminiferous tubules and cell degeneration (3). On the other hand, the gene expression of OCT4 was lower ($p<0.05$) in C-6h in relation to F-6h; however, the expression of C-KIT showed no differences ($p>0.05$) between fresh and frozen tissues after 2 and 6h of storage. Similar results were observed when cryopreserving testicular tissue of Black-Footed Ferret (4). OCT4 plays an important role in the proliferation and differentiation of spermatogonial stem cells, the increase in expression is induced by stress conditions during the cryopreservation process (5). It is concluded that the testicular tissue of prepubertal goats, after being stored for 6 hours (C-6h), presents a greater resistance to the cryopreservation process.

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Animal Reproduction

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MALE REPRODUCTIVE BIOLOGY

Cryopreservation effects on semen parameters, hatchability, and offspring development in captive North African Houbara bustard (*Chlamydotis undulata*)

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Among reproductive biotechnologies, artificial insemination, and semen cryopreservation, play a critical role in the success of wildlife conservation programmes. They enable the creation of semen banks for genetic preservation and optimisation of genetic management of captive populations. However, the effects of these biotechnologies on sperm quality and offspring development are not well understood. The conservation breeding program for the North African houbara bustard relies solely on artificial insemination, therefore, it is crucial to master semen analysis and cryopreservation techniques. The primary objective of this study was to assess the efficacy of cryopreservation in maintaining the functional characteristics of houbara sperm. This was achieved by analyzing the semen of adult males before and after cryopreservation. Semen was collected from adult males (n=50, age 4 ± 1.54 years) using a dummy female and samples were divided into two aliquots: one was analyzed and used for fresh insemination, and the second was cryopreserved, evaluated, and used after thawing. The semen samples were evaluated for motility and velocity, including total motile and curvilinear velocity, using the SCA system. Viability was assessed using propidium iodide and SYBR green. Sperm chromatin structure assay was used to determine DNA integrity and compaction (DFI% and HDS%) and compaction related to protamine content using CMA3. Additionally, the study aimed to investigate the impact of cryopreservation on fertility, embryonic death, hatching success, offspring growth (weight). Females (n=123, age 4± 1.53 years) were inseminated once, either with a fresh or a frozen/thawed sperm. Eggs (n=365) were collected and incubated. Hatched chicks weighted daily for 30 days. The sperm parameters were evaluated using paired T-test, and Wilcoxon signed rank test, depending on the distribution's normality. Generalized and linear mixed-effect models were run to assess the effect of cryopreservation on hatching success and chick growth rate, controlling for dependency between siblings, offspring age and sex, individual ID (chick, female, and male) for repeated measures. Results indicate that fresh samples had a higher percentage of motile sperm (88.94 ± 6.25%) compared to frozen samples (54.44 ± 13.38%), as well as higher VCL (42.21 ± 6.68µm/s versus 29.95 ± 5.94µm/s) and higher viability (90.91 ± 6.21% versus 56.29 ± 16.24%). In addition, the DNA damage was lower in fresh samples (2.83 ± 1.52% versus10.9 ± 7.76%). The sperm cell DNA was also more compact in fresh samples (10.5 ± 5.36%) compared to frozen samples (58.4 ± 12.6%), with a significant difference also observed in the CMA3 positivity (0.32 ± 0.46% and 1.71 ± 3.14% for fresh and frozen samples, respectively). Significant differences were observed in the rate of non-fertile eggs and early embryo death, with higher percentages when using frozen semen leading toa lower hatching success (58.56% for fresh and 47.83% for frozen). Finally, cryopreservation had no detrimental effects offspring growth rate up to 30 days. Despite observed impacts on sperm parameters, the hatchability remained acceptable, considering what is known for domestic species (40% in average), with no short-term impact on offspring development, underlying the value of such biotechnology applied to the conservation of this endangered species. However, based on literature, observed high DNA decompaction on cryopreserved sperm, call for further exploration on its potential long-term effects on offspring development.

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MALE REPRODUCTIVE BIOLOGY

STUDY OF THE INTERACTION BETWEEN SPHINGOMYELIN AND BSP 1 FROM BOVINE SEMINAL PLASMA USING COMPUTATIONAL BIOLOGY

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Binder of Sperm Proteins (BSP) are present in the seminal plasma of several mammalian species, with BSP1 being the most abundant found in cattle. BSP1 is a low molecular weight glycosylated protein containing two fibronectin type II domains (1) that allow specific binding to the phosphorylcholine of the sperm membrane. This binding during ejaculation induces the e-flux of cholesterol and phospholipids from the sperm membrane, a fundamental step in promoting sperm capacitation. In this context, the aim of this study was to use crystallographic data of the BSP complex (PDB ID: 1H8P), data from the National Library of Medicine (PubChem, https://pubchem.ncbi.nlm.nih.gov/) to obtain sphingomyelin and the computational methods of molecular docking, molecular dynamics (MD) and quantum biochemistry in order to decipher, at the molecular level, the interaction between BSP1 and sphingomyelin (SM), one of the main phospholipids that makes up the sperm membrane in cattle. The ProteinPrepare server, as well as MarvinSketch 18.24 and Discovery Studio 2020 were used to adjust the protonation of the molecules (BSP and SM) to pH 7.4. Molecular docking was carried out using AutoDock Tools 1.5.6 and AutoDock Vina 1.1.2. Next, the pose with the most attractive docking score was selected to carry out the molecular dynamics (100 ns) of the BSP::SM complex using GROMACS 2021.2. The last conformation of the molecular simulation (100 ns) was used to carry out the molecular fragmentation with conjugated caps and, subsequently, to carry out the interaction energy calculations based on density functional theory using the DMOL3 package in the Materials 8.0 software. The DM results indicated that the complex was not reasonably stable, which may be an indication that the interaction did not occur at the site indicated by the docking. However, the low stability may be related to the long hydrophobic chains of SM, which do not interact strongly with BSP1. Furthermore, quantum calculations indicated that the possible interaction of the complex may be mediated mainly by the following residues: TRP106, PRO27, PHE27, ARG104, PHE36, LYS107, ALA105, LYS34, LEU96 and VAL25. It is also worth noting that SPH established a hydrogen bond with ARG104. In conclusion, the results allowed us to understand how SM interacts with BSP1. It is possible to theorize that this phospholipid does not interact stably with the protein. Further studies will provide data to identify, by comparison, which residues may be related to the success of sperm capacitation.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

COMPARATIVE ANALYSIS OF MORPHOMETRIC PARAMETERS OF SPERM CELLS IN SMALL RUMINANTS

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This study aimed to make a comparative approach between the morphometric measurements of spermatozoa from sheep and goats. Seven sexually mature animals were used in this study, four male sheep and three of the goat species, with average weights of 58kg and 50kg, respectively, for each species. These animals were housed in individual pens with feeders and drinkers and subjected to intensive management. Ejaculates were obtained weekly from each ram, totaling ten ejaculates each week with the help of a conditioned female and an artificial vagina. Semen samples were sent to the Laboratory of Studies in Animal Reproduction (LERA/UFC), and each ejaculate was evaluated for volume (ml) and sperm concentration (×109 sptz/ml) using spectrophotometry. Subsequently, semen was diluted in TRIS to obtain a final concentration of 100×106 sptz/ml. Bromophenol Blue (AB), Eosin-Nigrosin (EN), and Rapid Panoptic (PA); in addition, unstained semen smears were made to evaluate standard cells (control). Images of 500 spermatozoa were digitized for each stain and for the control, totaling 2000 sperm cells analyzed per species. These cells were evaluated by ImageJ, a software for image processing and analysis. The following measurements (μm) were made: head length, width, perimeter, and area, tail length, and total spermatozoa length. The data were analyzed using the SAS statistical program (2009), subjected to ANOVA, and means were compared by Tukey's test at 5% significance level. The results of the morphometric analysis of spermatozoa revealed that the values in the ovine species are statistically superior ($P<0.05$) for all evaluated parameters. The values of morphometric measurements (μm) of length, width, perimeter and area of the head, tail length and total sperm length in sheep were higher in relation to those seen in goats, which suggests that the dyes have different effects on sperm cells in different species of small ruminants. It was concluded that the dyes used in this work induced an increase in morphometric measurements in sheep sperm and decreased the parameters of sperm cells in goats. Understanding the differences in sperm dimensions between small ruminants can contribute to improving semen evaluation methods in these species.

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MALE REPRODUCTIVE BIOLOGY

INFLUENCE OF DIFFERENT LEVELS OF FOOD RESTRICTION ON THE THICKNESS OF THE TESTICULAR SAC IN LAMBS

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Reproductive efficiency in sheep is influenced by intrinsic factors such as genetics, and extrinsic factors such as management, environment, and nutrition. Studies suggest that nutritional factors may be the most significant concerning productive processes, potentially mitigating the effects of other factors (1). In situations of low nutritional intake, animals tend to adjust, allowing anatomical and functional changes that increase the chances of survival and successful reproduction. Thus, the objective was to evaluate the scrotal circumference of sheep subjected to different levels of food restriction. Thirty prepubescent crossbred F1 Dorper x Santa Inês lambs with an initial body weight of 31.87 ± 0.5 kg and 157 days of age at the start of the experiment were used. The animals were distributed in a completely randomized design, in a 1 × 3 factorial scheme, with one sex class (30 males) and three feeding levels (ad libitum, 30%, and 70%). The sheep were kept under adequate sanitary conditions, vaccinated, dewormed, identified, and allocated to individual masonry pens (2 m²) equipped with feeders and drinkers. Scrotal Bag Thickness (SBT) was obtained using a caliper in the mid-scrotum region. The data were subjected to analysis of variance, and the means were compared using the Tukey test ($p < 0.05$) using the statistical program (R Development Core Team, 2017). EBE had higher values in ad libitum animals (0.37 ± 0.033A,c , 0.51 ± 0.033A,cb, 0.58 ± 0.033A,a and 0.70 ± 0.033A,a for 157, 173, 191, and 211 days, respectively) and at 211 days of age ($p<0.001$), with 70 ± 0.033A,a for the ad libitum treatment, 0.50 ±0.033B,a for T30, and 0.47 ±0.034B for T70. EBE was influenced by the treatments adopted in the present study, as animals subjected to food restriction did not show a significant increase in thickness ($p< 0.05$). However, for the ad libitum treatment, although no difference was observed ($p>0.05$) for the first three measurements, in the last one at 211 days, the difference was evident, emphasizing the importance of good nutrition. It is concluded that food restriction showed negative effects on scrotal bag thickness, not being advantageous to subject woolless lambs to restriction.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

EFFECTS OF FOOD RESTRICTION ON REPRODUCTIVE DEVELOPMENT IN DORPER X SANTA INÊS CROSSED LAMBS

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In Brazil, Sheep farming is one of the livestock farming activities that has been standing out in recent decades, therefore, comes up the necessity of choosing breeders prematurely, since then, the reproduction is one of the essential parameters in one production system, so that it is necessary to make a study to choose assertive and precocious way of sheep breeders. Given this scenario, the objective is evaluating the reproductive advantage of using F1 Dorper X Santa Inês crossbreeds at different restricted levels of restriction meals, evaluating the onset of puberty in relation to food supply and age group. Therefore, food was the main variable used to evaluate morphometric measurements, which are parameters for deciding whether or not the diet is favorable. Thirty prepubescent crossbred F1 Dorper x Santa Inês lambs with an initial body weight of 31.87 ± 0.5 kg and 157 days of age at the start of the experiment were used. The animals were distributed in a completely randomized design, in a 1 × 3 factorial scheme, with one sexual class (30 males) and three dietary levels (ad libitum, 30% and 70%). All the animals were dewormed, identified and allocated in individual stalls, equipped with a feeder and waterer. In the first two weeks, the animals were subjected to their respective diets, mounting training and ejaculation in the artificial vagina., in addiction to measuring a Scrotal Circumference (SC). There was measured the SC, a measuring tape was used, the testicles were pulled to the lower part of the scrotum, so that the measurement was carried out at the widest point of the scrotum. After measurements, each animal was subjected to semen collection using an artificial vagina and a mannequin (female sheep previously conditioned). Immediately after collection, the ejaculate obtained was analyzed according to prior methodology (1). SC was influenced by diet ($p<0.001$) and age ($p<0.001$) independently. Therefore, animals under food restriction had lower development and were greater in older animals. SC is an important parameter for judging the reproductive quality of the animal, reflecting indothe weight of the testicle and a sperm production. Regarding seminal analysis, few animals in the control group ejaculated throughout the experiment. However, none of the ejaculates contained sperm, therefore, none of the animals reached puberty at the end of the experiment, it is concluded that the reduction in food interfered with the growth of the testicles, as heavier males showed greater testicular development.

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MALE REPRODUCTIVE BIOLOGY

Global proteomics analysis of spermatozoa from locally-adapted rams

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The Brazilian Northeast holds 63.02% of the national sheep flock. Despite the predominance of extensive systems, reproductive biotechnologies are powerful strategies for enhancing and genetically improving herds. The combination of semen cryopreservation and artificial insemination is the most cost-effective tool, although individual variation among animals can influence outcomes. Studying plasma and sperm components related to successful freezing will enable greater control and selection of the best animals for cryobanks. "Shotgun proteomics" is an analysis strategy based on bottom-up proteomics, which detects and quantifies proteins in complex biological samples by breaking down proteins, separating the fragments, and analyzing them using mass spectrometry to identify peptides. The present study aimed to evaluate the sperm proteome of ovine breeders using the "shotgun" protocol for mass spectrometry and bioinformatics tools. To achieve this, a sexually mature, healthy ovine breeder kept in facilities with adequate roughage and concentrate supply and ad libitum access to water was used. Semen was collected 10 times at 3-day intervals using an artificial vagina previously heated to 37°C and in the presence of a female with induced estrus. The semen samples were immediately analyzed for kinetic parameters and then treated with a protease inhibitor. After each collection, the semen underwent centrifugation at 4°C and was washed three times with PBS to isolate the spermatozoa, which were then stored in a freezer at -20°C. A pool of sperm samples from the animal was made, then resuspended in a lysis buffer containing urea and sonicated on ice three times, followed by quantification of proteins using the Bradford colorimetric method[1]. After quantification, excess urea was removed, and the proteins underwent trypsin digestion and were neutralized with trifluoroacetic acid (TFA). Desalting of the peptide sample was performed using SepPak TC18 columns installed in a vacuum system. Then, 1 µg of trypsin-digested peptides was analyzed using a chromatographic system of capillary columns coupled to a quadrupole-orbitrap hybrid mass spectrometer. The identification and selection of peptide spectrum matches were conducted using the Comet software integrated into PatternLab for Proteomics (Version V). A total of 787 proteins were identified through sequences obtained from the Ovis aries proteome from UniProtKB (with 23,108 proteins), with the most abundant ones being beta tubulin chain, proteins containing amine oxidase domain, beta subunits of ATP synthase, glyceraldehyde-3-phosphate dehydrogenase, interleukin 4-induced, alpha tubulin chain, proteins containing THAP domain, alpha subunits of ATP synthase, clusterin, and phosphoglycerate kinase. Genetic ontology analyses were performed using the String databases, revealing the greatest enrichments in groups of genes out of a total of 486 unique identified genes. Regarding the biological processes involved, the top 5 clusters are related to cellular processes, metabolic processes, cellular metabolic processes, organization of cellular components, and organization of organelles. The clusters with predominant molecular functions are related to catalytic activity, ion binding, binding of cyclic organic compounds, binding of heterocyclic compounds, and binding of small molecules. Regarding the cellular component, the enriched groups are mainly located in the cellular anatomical entity, intracellular anatomical structure, organelles, cytoplasm, and intracellular organelles. The analysis using shotgun proteomics demonstrated highly promising results, revealing significant depth in protein identification even in more complex samples. It was also possible to identify proteins related to cellular protection against cold, such as heat shock proteins (HSPs), as well as pathways related to cellular protection mechanisms, mainly against apoptosis. It is known that locally adapted, unshorn rams possess proteins with roles in sperm protection, as demonstrated in this study[2]. Considering the ability to comprehensively address the proteome provides insights into complex biological systems, this technique promises to continue playing a fundamental role in understanding protein expression and advancing biomedical research.

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X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Effect of freezing extenders on post-thaw sperm motility of quarter horse stallions' semen after short-cooled storage

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Among other customizable uses, freezing semen after cooled storage allows practitioners (1) to collect stallion's ejaculates at stud farms and transport cooled samples to the reproduction laboratory for further processing, (2) to freeze received doses arriving too late after the mare's ovulation instead of throwing them away, and (3) to freeze extra doses received during the off-season to establish a semen bank on the farm, thereby avoiding the lack of semen availability during the peak of the breeding season for busy stallions. There are different freezing protocols available, including various curves and centrifugation methodologies. However, stallions are selected for semen cryopreservation based on performance and pedigree, regardless of their fertility or freezing ability. Strategies that allow individual improvement of their reproductive performance are important and few consider a late test freeze to determine the best extender matching for each stallion using a single manual curve that can be performed under field conditions. Thus, the aim of the present study was to assess the impact of different freezing extenders on post-thaw sperm quality after cooled storage. One ejaculate from each of ten mature Quarter Horse stallions, continuously used in a reproduction center for inseminations and semen freezing, was collected. Ejaculates were diluted with EquiPlus (Minitube, Germany) up to 50 million sperm/ml before storage at 5°C for 8 hours. After storage, samples were divided into nine centrifuge tubes and centrifuged for 20 min at 600g force. The supernatant was then aspirated and discarded, and the sperm pellet was resuspended in each tube with a different freezing extender. Following resuspension, the identified straws were manually filled and sealed before being kept at 5°C for 20 min in a refrigerator. Subsequently, they were exposed to nitrogen vapor for another 20 min on a floating rack before immersion in liquid nitrogen. After the freezing process, the straws were thawed in a water bath at 37°C for 30 seconds. Evaluations of sperm total motility (TM) and progressive motility (PM) after cooling storage (t0) and after thawing (t1) were performed using the CASA System (AndroVision, Minitube Germany). These parameters were compared between extenders by one way ANOVA, following Bonferroni test. To analyse the number of samples that showed post-thaw PM ≥ 30% according to the extender, a generalized linear model was used. Differences were considered significant when P values were <0.05. Values are expressed as mean ± SD (Graph Pad, USA). Mean sperm TM and PM decreased from t0 to t1(TM: 71.4±9.7% vs. 31.8±11.3% and PM: 66.8±12.3% vs. 24.8±10.3%) (P<0.0001). Post-thaw TM did not show significant differences between extenders, except for samples diluted in Violet and Star, whose TM was greater than for Blue (P=0.026). Post-thaw PM was significantly greater in samples diluted in Star, Violet and Botucrio vs. Blue (34.8±4.9%, 34.3±8.7% and 32.5±8.5% vs. 18±8.5%, respectively) (P= 0.01). An effect of the extender on the proportion of samples that showed post-thaw PM>30% was observed (P<0.0001). This proportion was significantly greater for Violet than for Blue, MX3 and Orange (P=0.04), and for Star than for Blue (P=0.04). Considering the stallion's particularities regarding freezing performance, only one did not reach a post-thaw PM>30% with any extender, while most stallions were approved in at least two protocols (extenders). Besides, 2 males were approved in 6 of 9 extenders tried. Focusing on the best PM obtained for each approved stallion, 4 of them performed better with Violet, 2 with Botucrio, while only one with each MX3, Gent, Star and Red. On the contrary, any stallion attained its best PM with Green, Orange, or Blue. The 80% of samples diluted in Star and Violet (8/10), and 60% of those diluted in Botucrio (6/10) showed a post-thaw PM ≥ 30%, while only 10-30% of samples diluted in the other extenders, did. However, no extender failed in all stallions. In conclusion, Quarter Horse semen diluted with EquiPlus can be frozen after short storage, resulting in approved straws. Violet emerged as the safest extender choice using this freezing protocol, as it yielded more approved samples and achieved the highest post-thaw motility in three stallions, followed by Star and Botucrio. However, the test-freeze demonstrated that to achieve the best post-thaw motility possible in each stallion tested, six extenders were required. This proves that protocol customization is mandatory when the goal is to obtain the best results after thawing, whereas relying on a single extender is not sufficient.

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MALE REPRODUCTIVE BIOLOGY

INFLUENCE OF DIFFERENT DYES ON THE MORPHOLOGY OF SPERM CELLS IN SMALL RUMINANTS

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Sperm morphology constitutes a key factor in mammalian reproduction, which, in turn, relies on the quality of germ cells (1). Thus, sperm morphology is arguably the best source of information regarding male fertility (2). The aim of this study was to investigate whether different types of dyes used promote alterations in the morphology of sheep and goat sperm cells. Six sexually mature animals were used, three of each species, with average weights of 58kg and 45.8kg, respectively, for each species. Semen collections were performed weekly, and ejaculates were obtained using artificial vaginas and conditioned females. A total of ten ejaculates were obtained from each species, which were evaluated for volume (×109 sptz/ml) and then diluted in TRIS. Three aliquots were taken from each ejaculate to prepare smears, which were stained with Bromophenol Blue (AB), Eosin-Nigrosin (EN), and Rapid Panoptic (PO). For each dye, ten semen smears were made, and 200 sperm cells/slide were evaluated, totaling 2000 spermatozoa analyzed per dye. After drying, the slides were viewed under a light microscope at a magnification of 1000×, and sperm images were digitized. Sperm cells were classified as Normal (NOR), and morphological alterations were classified as Major Defects (DEMA) and Minor Defects (DEME). NOR, DEMA, and DEME data were subjected to ANOVA using the SAS statistical program. Means were compared by Tukey's test at 5% significance level ($P < 0.05$). In sheep, the highest value for DEMA was observed under the influence of PO staining (44.10a), although it did not statistically differ from AB (31.20ab). Regarding morphologically normal spermatozoa, the highest number was observed with EN dye (166.40a), but it did not significantly differ from AB (155.70ab). For both species, no effects of dyes on DEME values were observed ($P > 0.05$). In goats, the highest value for major defects was observed with EO dye (52.10a), while AB (29.80b) and PO (35.40b) did not statistically differ from each other. There was an increase in the number of normal spermatozoa with AB (152.40a), although no significant differences were observed when compared to PO (139.80ab). However, EO (130.50b) showed lower values ($P < 0.05$) for normal spermatozoa compared to AB. It was concluded that the dyes Bromophenol Blue, Eosin-Nigrosin, and Rapid Panoptic differently alter the sperm morphology of sheep and goats. In this study, it was found that EO dye was detrimental to the morphological characteristics of goat semen, while among the dyes used, it is the most recommended for morphological evaluation of sheep semen. Therefore, further studies are necessary to investigate how these dyes interact with spermatozoa, in addition to using a wider variety of staining methods in the semen of both species to determine the dye that best preserves the morphological characteristics of sperm cells.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Sperm kinetics of fresh and frozen-thawed semen: defining a protocol for cryopreservation and parameters for rams with low and high semen freezability

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The aim of this study was to establish a cryopreservation curve for semen and to define parameters for selecting rams with higher semen freezability. Twelve adult hair rams were selected, fed with Tifton hay (Cynodon dactylon), concentrate, and mineral supplement, and subjected to six semen collections at 72-hour intervals, using artificial vagina. After each collection, an aliquot of fresh semen was diluted (1:100) and evaluated with a portable device (Computer-assisted sperm analyzer; AndroScope®, Minitube, Germany) for total motility (TM), progressive motility (PM), curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP). After each collection, spermatozoa were diluted in cryopreservation medium (Optidux®, Reprodux, Brazil) and packaged in 0.25 mL straws (100 x 10^6 spermatozoa). Cryopreservation was performed using an automated machine (HSE 750, NeoVet, Brazil) with a curve that, in the positive ramp, decreased by 0.3°C/min until reaching 4°C, where it remained stabilized for 4 hours. Then, the negative ramp began, with a decrease of 5°C/min at the first stage until reaching - 20°C, and a decrease of 20°C/min at the second stage. Upon reaching -110°C, the straws were immersed in liquid nitrogen. After 7 days, the straws were thawed at 37°C for 30 seconds to evaluate seminal parameters. The data were subjected to normality and homoscedasticity tests. The means of TM and PM for fresh semen were 89.1 ± 6.3% and 85.4 ± 6.8%; for VCL, VSL, and VAP, they were 212.8 ± 20.9 µm/s, 99.1 ± 17.2 µm/s, and 114.4 ± 16.8 µm/s, respectively. In the case of frozen-thawed semen, parameters were 56.4 ± 17.7% and 49.5 ± 16.9% (TM and PM) and 138.9 ± 22.8 µm/s, 47.7 ± 9.1 µm/s, and 61.2 ± 10.3 µm/s (VCL, VSL, and VAP). Total motility reduction (TMR) was calculated as the difference between TM of fresh and frozen-thawed semen. TMR median (31.6) was used to distinguish semen freezability of the rams. Animals (n = 6) with higher cryopreservation potential (HPC) had TMR < 31.6, while animals (n = 6) with lower cryopreservation potential (LPC) had TMR ≥ 31.6. The variables measured in HPC and LPC groups were subjected to Student's t-test. There were no differences ($p > 0.05$) in fresh semen TM and PM for HPC and LPC rams. However, TM and PM values in thawed semen showed differences ($p < 0.01$) between HPC animals (62.7 ± 13.9% and 55.5 ± 13.4%) and LPC animals (50.2 ± 18.9 and 43.4 ± 17.9). VCL, VSL, and VAP values did not show significant differences in fresh or thawed semen between groups ($p > 0.05$). This study establishes a protocol and the feasibility of the cryopreservation curve for hair sheep. Animals with the same quality of fresh semen showed significant variations in sperm cryoresistance. The reasons for these variations are still unknown but may be related to the molecular composition of seminal plasma, metabolism, and energy of sperm cells.

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MALE REPRODUCTIVE BIOLOGY

Quantitative proteomic analysis of penile squamous cell carcinomas and associations with human papillomavirus (HPV) infection status

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Penile cancer (PCa) accounts for nearly 10% of all neoplasms in men in certain regions of Asia, Africa and Latin America. Brazil has one of the highest incidences of PCa and most cases are diagnosed in the North and Northeast regions. Risk factors for PCa include poor hygiene, HPV infection, sexual behavior, chronic inflammation of penile skin and phimosis. PCa is aggressive and treatments are limited, frequently requiring penectomy, and molecular signatures of PCa are largely unknown. Penile cancer is considered a neglected disease. The present study describes a comprehensive proteomic analysis of penile squamous cell carcinoma from patients with distinct HPV infection status. Patients (62.8 ± 3.8 years) were diagnosed at the Cancer Institute of Ceará (ICC) and tumor and non-tumor samples, collected at the time of penectomy. The study was approved by ICC Ethics Committee. Patients were tested for HPV using immunohistochemistry (p16INK4a expression) and in situ hybridization. Proteins extracted from penile tumor (n = 16) and non-tumor (n = 8) samples were subjected to trypsinization, desalting and label-free mass spectrometry. Spectra files were analyzed by Xcalibur (Thermo Scientific, USA) and mass spectrometry data, using MaxQuant. Peptide abundances were exported to pmartR package for quality control and statistics. Protein networks, functional clusters and pathways were analyzed by Cytoscape's App. There were 147 proteins identified exclusively in tumor and 112 proteins, in non-tumor samples, with 514 shared proteins. Tumor samples came from 8 patients with high-risk HPV and 7 patients without this condition. There were 48 proteins exclusive to high-risk HPV patients, 130 proteins exclusive to patients with no HPV, and 675 common molecules. Comparative analyses of samples coming from patients with (ME+, n = 6) or without (ME-, n = 8) lymph node metastasis indicated 250 proteins in ME- patients, 21 proteins in ME+ patients, with 573 shared proteins. Categorization of samples based on disease free survival (DFS) showed that 55 proteins were exclusive to patients with DFS > 17.5 months (n = 6), 191 proteins exclusive to patients with DFS < 17.5 months (n = 7), with 619 shared proteins. Genes coding for proteins with differential abundances ($p < 0.05$) in penile squamous cell carcinoma and non-tumor tissues were associated with tricarboxylic acid (TCA) cycle, respiration electron transport, and glucose metabolism, genetic information processing, cell cycle, angiogenesis and metastasis. Altered proteins in tumor tissues from patients with HPV were associated with lipid metabolism, TCA cycle, glucose metabolism, chromatin regulation, cell cycle and immune response, among other events. Tumor samples from patients diagnosed with and without lymph node metastasis exhibited distinct proteome landscapes and altered pathways as well, including RNA processing and splicing, ribosome, and nucleotide metabolism, immune response, complement and coagulation, cell cycle. Molecules associated with lipid and glucose metabolism, such as apolipoprotein, catechol-O-Methyltransferase, fatty acid binding protein 5, phosphoglycerate mutase 1 and aldolase A were up-regulated in tumor samples from patients with DFS >17.5 months. Additionally, proteins related to RNA processing/splicing, ribosome and translation factors, microtubules and cytoskeleton organization, angiogenesis and metastasis were altered in patients with DFS greater than 17.5 months. In conclusion, this is the first high-throughput proteomic approach for penile cancer research in Brazil. Major alterations in the proteome atlas of penile squamous cell carcinomas related to vital pathways controlling energy metabolism, genetic processing, cytoskeleton structure, cell cycle, and immune responses. HPV infection appeared to inflict significant modifications in the proteome of penile tumors as well. Molecules with altered expressions in the penile tumors will serve as potential targets for drug screening, diagnostic strategies and therapy.

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X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Effects of different types of serum albumin on function, kinetics and capacitation of ram spermatozoa

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Sperm capacitation involves physiological and biochemical changes [1, 2]. In the female genital tract, it is a gradual, coordinated, and progressive process [3, 4]. In vitro, it can be reproduced by incubating spermatozoa in a medium that mimics the oviductal fluid [5, 6]. Currently, bovine serum albumin is extensively studied in the context of in vitro sperm capacitation [7, 8, 9]. However, there is a lack of information about the effects of different types of albumins on sperm capacitation in rams. Understanding these effects will have significant implications for assisted reproduction in rams and other economically relevant species. Thus, this study aims to analyze the function, kinetics, and in vitro capacitation of ram spermatozoa using bovine, human, ovine, and caprine serum albumins (BSA, HSA, OSA and CSA, respectively). Ten mature, healthy, and fertile Assaf rams were selected. Semen collection was performed over two consecutive days, obtaining a single ejaculate from each male. Subsequently, the samples were assessed for volume, mass motility, and sperm concentration, diluted at a 1:1 ratio, and divided into five distinct experimental groups. The experimental groups consisted of sperm capacitated in TALP medium with no albumin (NA; control group), and sperm capacitated in TALP medium supplemented with BSA, HAS, OSA and CSA. Sperm motility, kinetics and cellular functionality were evaluated for all treatments. Prism 9 software (GraphPad Software, San Diego, CA, EUA) was used to analyze the data. Significant differences were considered with $p < 0.05$, and the data were subjected to Shapiro-Wilk tests for normality. The analysis was conducted by one-way ANOVA, or Kruskal-Wallis for non-normally distributed data. Treatment OSA had significantly decrease both in total and progressive motility compared to the other treatments, indicating an adverse effect of ovine serum albumin on ram spermatozoa. Treatment NA demonstrated the highest viability, being significantly distinct only from OSA. However, the treatment with OSA showed the highest value among all treatments in the analysis of reactive acrosomes, significantly differing from the treatments with NA and BSA. Regarding capacitation state analysis, all treatments with albumins showed similarity among themselves and distinction from treatment NA. No significant differences were observed between treatments regarding parameters of FPM, VCL, LIN, ALH, apoptosis, and mitochondrial functionality. Based on these results, we observed a significant distinction between OSA and BSA. This variance may be attributed to OSA's potential affinity with receptors present in ram spermatozoa, as well as its biochemical properties that could trigger the necessary changes for sperm capacitation more efficiently. This could result in a rapid loss of sperm motility and viability, leading them to enter a capacitated state more promptly [10, 11, 12]. Factors such as the ability to affect the sperm membrane, production of reactive oxygen species, and removal of inhibitory proteins may influence the effectiveness of OSA in promoting in vitro sperm capacitation [13, 14, 15, 16]. However, further study is needed to better explain this phenomenon.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Effect of crude cajá gum on bovine semen cryopreservation

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Cryopreservation of seminal fluid causes a reduction in sperm viability due to the formation of intra- and extracellular ice crystals. Among the compounds that have the potential to neutralize these deleterious effects, crude cajá gum was considered an extracellular cryoprotective substance. The aim was therefore to evaluate the effect of crude cajá gum on the cryopreservation of bovine semen. Crude cajá gum was collected from the natural exudates of the cajazeiro tree. In the isolation stage, as suggested by Rodrigues et al (1993), the crude gum was separated from most of its impurities and its acid constituents neutralized. Two purification steps were then carried out to replace the cations present in the gum, before it was freeze-dried. Twenty-one normospermic ejaculates were collected by electroejaculation and diluted in Tris-Gema medium in five treatments: Control (Tris-yolk + 6% glycerol), Treatment 1 (T1 - Tris-yolk + 3% glycerol), Treatment 2 (T2 - Tris-yolk + 0% glycerol), Treatment 3 (T3 - Tris-yolk + 0.5% crude cajá gum), Treatment 4 (T4 - Tris-yolk + 1.5% crude cajá gum), and Treatment 5 (T5 - Tris-Gema + 5.5% glycerol + 0.5% crude cashew gum), and in 0.25ml straws, with a concentration of 40 million sperm per straw, were frozen in liquid nitrogen and stored in a cryogenic cylinder. The Shapiro-Wilk test was used to test the normality of the data. An ANOVA test was used to compare different treatments. Duncan's test was used to find the difference between treatments. The level of significance was set at $P \leq 0.05$. Data is presented as mean ± standard error. The means and standard error of the percentage of total motility and sperm vigor were significantly ($P \leq 0.05$) higher in the control and Tris-Gema + 3% glycerol (T1) treatments than in all the other treatments. The treatments supplemented with Tris-Gema + 0% glycerol (T2), Tris-Gema + 0.5% crude cajá gum (T3), Tris-Gema + 1.5% crude cajá gum (T4) showed lower ($P \leq 0.05$) total sperm motility and sperm vigor than all the other treatments. The treatment containing Tris-yolk + 5.5% glycerol + 0.5% crude cashew gum (T5) showed significantly higher total sperm motility and sperm vigor ($P \leq 0.05$) than those with Tris-yolk + 0.5% crude cashew gum (T3), Tris-yolk + 1.5% crude cashew gum (T4). The mean and standard error of sperm tail defects was significantly ($P \leq 0.05$) higher in the control and Tris-Gema + 3% glycerol treatments. However, treatment 1, supplemented with 3% glycerol, did not differ from the treatments supplemented with cashew gum. The highest average total defects were observed in the control treatment compared to all the other treatments. Cashew gum is water-soluble and acts as a gelling agent, thickener, stabilizer and coating agent, justifying the preservation of sperm morphology. In conclusion, the addition of cashew gum to bovine semen diluent, alone or in combination with glycerol, did not improve total motility and sperm vigor, but did reduce sperm tail defects and total defects. However, more research is needed to fully understand the effective concentrations, the appropriate mechanism and the benefits and limitations of cashew gum in the cryopreservation of bull semen.

Keywords: Andrology, seminal evaluation, freezing, diluent, gum.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Proteomics of ram seminal plasma Morada Nova

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Seminal plasma secreted from the epididymis and accessory sexual glands has an important role in modulating sperm physiology. This work aimed to characterize the proteome of the seminal plasma of Morada Nova ram. To this end, the semen was collected using an electroejaculator and the seminal plasma was separated from the sperm cells through centrifugation. Proteins of seminal plasma were precipitated, digested with trypsin, desalted and subjected to LC-MS/MS. The peptide spectrum search carried out using the PatternLab V software allowed the identification of 944 proteins in seminal plasma, including clusterin, peptide YY, spermadhesin Z13-like, lactoperoxidase, EGF like repeats, binder of sperm 1, glyceraldehyde-3-phosphate dehydrogenase. Of the proteins observed, clusterin is linked to the differentiation of germ cells in the process of reabsorption of defective sperm and modulation of cell lysis (1), spermadhesin has an important role in fertilization by helping to bind sperm to the oocyte (2), lactoperoxidase acts to protect sperm against oxidative stress (2) and binder of sperm 1 stabilized the membrane and qualitatively reduced tyrosine protein phosphorylation, but significantly increased cholesterol efflux and induced spontaneous acrosome reactions (3). The list of proteins was analyzed on the String 11.0 platform (http://string-db.org) for in-silico analysis based on predictions collected from direct (physical) data or indirect (functional) associations of the proteins (4). The most important biological processes associated with ovine seminal plasma proteins were identified as glyceraldehyde-3-phosphate metabolic process, positive regulation of telomerase RNA, positive regulation of early endosome to late endosome transport, positive regulation of establishment of protein localization to telomere and 5-phosphoribose 1-diphosphate biosynthetic process. The major molecular function linked to the seminal proteins related to p-type sodium: potassium-exchanging transporter activity, l-lactate dehydrogenase activity, succinate-CoA ligase (ADP-forming) activity, proteasome-activating activity and threonine-type endopeptidase activity. Regarding the local network cluster (String), the threonine protease, chaperonin TCP-1, Fructose-1,6-bisphosphatase, cAMP, and cAMP-dependent catalytic protein kinase, alpha and beta tubulin were observed; KEGG pathways were highlighted as the most representative pathways: proteasome, pentose phosphate pathway, glycolysis/gluconeogenesis and citrate cycle (TCA cycle); in Reactome Pathways the main pathways were beta oxidation of palmitoyl-CoA to myristoyl-CoA, insulin effects increased synthesis of xylulose-5-phosphate, 5-phosphoribose 1-diphosphate biosynthesis, breakdown of the nuclear lamina, packaging of telomere ends. This represents a comprehensive atlas of proteins expressed in ram seminal plasma and will contribute to future identification of biomarkers related to ram fertility.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Comparison of three commercial extenders for the cryopreservation of Alpaca (*Vicugna pacos*) semen

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The artificial insemination (AI) in South American camelids has not yet reached its full development, partly due to the limited efficacy of standard procedures for semen cryopreservation. In alpacas, unsatisfactory results have been obtained due to marked variability in semen characteristics, collection methods, high viscosity, and diluents. Consequently, the objective of this study was to evaluate the effect of three commercial extenders on the cryopreservation of alpaca sperm. Thus, six fully mouthed breeders from the Canaán Agricultural Experimental Station of the National Institute of Agricultural Innovation (INIA), Peru, located at 2735 meters above sea level, were used. Animals were healthy and fed with natural pasture. Forty-one collections were conducted every two days using an artificial vagina incorporated into a dummy. Macroscopic aspects such as color, volume (mL), consistency (cm), and foam (cm) were evaluated, as well as microscopic aspects including motility (%), concentration (× 106 sperm/mL) using CASA software, and viability (%) with the use of an optical microscope. After collection, the ejaculate was diluted at a ratio of 1:1 using the three extenders (Optixcell, Steridyl, and Andromed), brought to room temperature for 10 min, cooled for 2 h, frozen, and subsequently thawed at 37°C. According to the distribution of data, these were evaluated by Kruskal-Wallis and Mann-Whitney tests using SPSS software with 5% confidence. The average mating time was 19.1 ± 4.9 min. The predominant color was milky in 75.6% of samples, semi-milky in 12.2%, and transparent in 12.2%. Fresh results for volume, consistency, and foam parameters were: 1.9 ± 1.4 mL, 1.4 ± 1.6 cm, and 2.2 ± 2.3 cm, respectively. Microscopic analysis yielded values of 77.85 ± 10.24% for motility, 112.3 ± 48.75 × 106 sperm/ml for concentration, and 80.6 ± 10.3% for viability. Motility results after cooling were 68.49 ± 10.01%, 59.51 ± 14.4% and 56.34 ± 14.53% for the Optixcell, Steridyl, and Andromed extenders, respectively, with the lowest ($P < 0.05$) percentages obtained by Andromed extender. Post-thaw motility was 39.83 ± 10.86%, 24.6 ± 10.02%, 27.27 ± 12.84% for the Optixcell, Steridyl, and Andromed extenders, respectively, and post-thaw viability was 49.61 ± 12.58%, 39.56 ± 13.32%, 36.85 ± 11.85% for the Optixcell, Steridyl, and Andromed extenders, respectively, with statistical differences found with Optixcell ($P < 0.05$). Given the limited studies on the individual impact of each preservation factor (such as dilution rate, extender type, and cryoprotectant), except for viscosity reduction treatment, the protocols used for alpaca semen cryopreservation are similar to those used for bull semen. Optixcell is composed of liposomes, whose activity is attributed to lipid molecules and cholesterol, which act on the sperm phospholipid membrane; thus, it could improve the results of semen cryopreservation. In conclusion, it can be stated that Optixcell provides a suitable microenvironment for sperm, which should result in better fertilization rates after AI. However, in vivo experiments must be carried out to verify pregnancy and birth rates.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Analysis of the effects of dyes on sperm morphology in sheep and goats

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Analysis of the morphological structure of sperm is an important factor that must be considered when determining the quality of a reproducer's semen, and it is also an important criterion for predicting fertility in males. During the analysis of sperm morphology, variations in sperm size, appearance and shape are found within the same species and even within the same ejaculate. The aim of this study was to analyze the influence of three dyes on the morphology of ovine and caprine semen. This experiment used three sheep and three goats that were whole, sexually mature and with body weights of approximately 58 and 45.8 kg, respectively. The ejaculates were collected using an artificial vagina and a conditioned female. Ten ejaculates were obtained from each species and three aliquots were taken from each ejaculate, each one stained with a different dye, totaling ten slides/dye for each species. In this study, the dyes used were Bromophenol Blue (AB), Eosin-Nigrosin (EO) and Rapid Panoptic (PO). The sperm were viewed under an optical microscope at 1,000× magnification and then scanned. 200 sperm cells were observed per slide, resulting in 2,000 sperm/colorant/species. The cells were classified as normal (NOR) and the morphological alterations as major defects (DEMA) and minor defects (DEME). The NOR, DEMA and DEME data were submitted to ANOVA using the SAS statistical program. The means were compared using the Tukey test at 5% significance ($P< 0.05$). In the analysis of the breakdowns of the interactions between dyes and species, it was possible to observe significant differences for the DEMA parameter only for EO between goats (52.10A) and sheep (25.40B), with a prevalence of morphological alterations in goats. No statistical differences were identified for the AB and PO dyes when only the species factor was considered ($P>0.05$). The number of normal sperm showed a statistical difference between the species, with the highest value in sheep semen (166.40A). For NOR, there were also no statistical differences for the AB and PO dyes when only the species factor was taken into account ($P> 0.05$). In goats, the dyes altered the morphology of the sperm cells differently, with the following DEMA values being obtained in AB (29.80b), EO (52.10a) and PO (35.40b). For sheep, the DEMA values obtained were 31.20b, 25.40b and 44.10b for the respective dyes. For goats, the AB (152.40a) and EO (130.50b) dyes showed a statistical difference in NOR, in which the AB dye better preserved sperm morphology. With regard to sheep, the values found for EO (166.10a) showed better preservation of sperm morphology compared to PO (143.00b), taking into account the NOR parameter. In this study, the EO dye was detrimental to the morphological characteristics of goat semen, with a high incidence of Major Defects. In sheep, EO was the dye that best preserved the normal morphology of the semen, while PO was detrimental to morphology and overestimated the Major Defects in the sperm cells of this species. It was therefore concluded that the dyes alter sperm morphology in different ways, and there may even be differences between species, as observed in this study.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Selection of spermatozoa from sheep in a microfluidic device: influence of chemotaxis and cold atmospheric plasma

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Microfluidics is a technology with potential applications in several areas, including reproduction. The advancement and utilization of microfluidic devices enable the precise evaluation of sperm behavior, facilitating the selection of the highest quality sperm for various reproductive techniques. Although this technology has already been tested in several species, no studies involving sheep have yet been conducted. Associated with this, studies on sperm selection also investigate the use of cold atmospheric plasma (CAP) to modify the surface of microfluidic devices, imparting a hydrophilic character to the material surface or facilitating the adhesion of PDMS devices to glass surfaces. However, to date, the influence of CAP application on sperm selection has not been evaluated. The objective of this study was to use microfluidic devices for the selection of sheep spermatozoa and to assess the impact of chemotaxis and cold atmospheric plasma on sperm cells. For this, a microfluidic device, consisting of an inlet reservoir and three outlet reservoirs, was manufactured using polydimethylsiloxane (PDMS). A total of 10 testicle-epididymal complexes from mature sheep were used. The sperm samples were divided into two groups: a control group consisting of devices that did not undergo any modification, and a treatment group in which the devices were exposed to atmospheric plasma for 15 minutes before selection. Subsequently, each outlet reservoir of the devices was filled with a different type of medium, namely in vitro fertilization (IVF) medium, 0.9% NaCl solution, and in vitro maturation medium (IVM). After filling, the sample containing spermatozoa was deposited in the inlet reservoir and the devices of the control and treatment group were incubated in an atmosphere of 37.5ºC and 5% CO2 for 35 minutes. After this period, the inlet reservoir was isolated and samples from the three outlet reservoirs and the inlet reservoir were collected for evaluation. As a control method for sperm selection, the Percoll gradient was performed. Sperm kinetic parameters were evaluated using Computer Assisted Sperm Analysis (CASA). Morphology was performed with bengal rose, membrane integrity from the hyposmotic test, and mitochondrial viability and activity by fluorescence microscopy. Data were expressed as mean ± standard deviation values. Statistical differences were verified by Analysis of Variance (ANOVA) followed by Tukey's test. Differences were considered significant when $p < 0.05$. The use of CAP did not influence the sperm quality parameters of sheep after the selection of epididymal spermatozoa, as the means for the parameters of motility, viability, plasma membrane integrity, and morphology showed no significant difference compared to the control group ($p > 0.05$). Regarding chemotaxis, the IVF medium showed higher total motility in relation to the 0.9% NaCl solution and higher progressive motility in relation to the IVM medium, both in the control group and in the treatment group ($p < 0.05$). This indicates that the IVF medium is more attractive to sperm and can be used for selection in microfluidic devices. This can be confirmed because the spermatic parameters of the sperm selected through the IVF medium in the microfluidic device were similar to those of the Percoll gradiente ($p > 0.05$). The substances present in the IVF medium provide acceleration of sperm capacitation and hyperactivation of sperm, which usually leads to an increase in the percentage of spermatozoa with progressive motility. Because of this, the IVF medium may have presented higher values in most of the kinetic parameters evaluated by CASA. The development of new techniques, such as microfluidics, for sperm selection with greater viability and integrity may be an alternative to achieve better fertilization rates in biotechniques such as in vitro embryo production. The evolution of more research in the area is essential to achieve these results.

Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Evaluation of pequi oil (*Caryocar coriaceum* Wittm) on intracellular levels of reactive oxygen species (ROS) in ram cryopreserved spermatozoa

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Semen cryopreservation is an essential technique in animal reproduction, allowing to improve semen use and transportation. However, this process can cause damage to sperm cells, resulting in a reduction in their fertilization potential, which is partially attributed to oxidative stress caused by the formation of reactive oxygen species (ROS). Ram spermatozoa is high susceptibility to oxidative stress during cryopreservation due to the membrane lipid composition, particularly to the high ratio of polyunsaturated fatty acids (PUFA) and low cholesterol to phospholipid ratio. The oil from pequi fruit (Caryocar coriaceum Wittm), due to its antioxidant action, appears as a potential additive in diluents for semen conservation. Thus, the aim of the study was to evaluate the addition of different concentration of pequi oil (PO) on intracellular levels of ROS in ram cryopreserved spermatozoa. Six Santa Inês ram breeders were used, and four ejaculates were obtained from each one (n=24). Each sample was subjected to dilution in powdered coconut water diluent (ACP®), added to 15% of egg yolk and 6% of glycerol and different concentration of PO (T0: 0%; T1: 5%; T2: 10%) and then cryopreserved in liquid nitrogen (−196°C). After 1 week, samples were thawed and evaluated for intracellular ROS levels. For the analysis, thawed samples were evaluated for intracellular oxidative stress using epifluorescence microscopy (400×; Episcopic Fluorescent Attachment EFA Halogen Lamp Set. Leica. Kista, Sweden), using the marker 2', 7'-dichlorofluorescein diacetate (H2DCFDA) (Thermo Fisher Scientific, USA). After incubation with markers, samples were analyzed, and sperm images were obtained in an epifluorescence microscope to determine the percentage of viable spermatozoa with intracellular ROS. The intensity of the fluorescence of the images was quantified using the ImageJ 1.45 s software (National Institutes of Health, Bethesda, Maryland, USA). The background signal intensity was subtracted from the values obtained for the treatment images. From each sample, up to 100 sperm were selected for the quantification of fluorescence intensity. The control group (0% PO) were used to calibrate the measurements, and the measured value of each sperm was divided by the mean of the calibrator to generate relative expression levels (1). The design adopted was completely randomized, with analysis of variance (ANOVA) and Tukey's test to compare means ($P<0.05$). It was observed that treatments added to pequi oil, irrespective of the concentration (T1: 0.78±0.25; T2: 0.76±0.24), had lower ROS levels compared to T0 (1±0), indicating its antioxidant action. There were no differences ($P>0.05$) among PO concentration used. This positive effect is attributed to the pequi oil composition, which includes vitamins E and C, phenolic compounds, and carotenoids, all recognized for their antioxidant properties (2). In addition, it contains large amounts of fatty acids, especially oleic, palmitic, myristic, palmitoleic, stearic, linoleic, arachidonic, heptadecanoic and eicosanoic, that potentially contribute for sperm motility and viability preservation after frozen-thawing process (3), therefore, PO acts as an additional source of these fatty acids, allowing the reduction of intracellular ROS formation. Thus, it was concluded that addition of 5 or 10% of PO to ACP® diluent is effective to the reduction of intracellular ROS formation during ram spermatozoa cryopreservation.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Analyzing the possible effects of different dyes on the head area and total length of sperm

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Sperm morphology, concentration, vigor, and motility are the most important parameters for semen analysis. Among these parameters, sperm morphology is one of the best ways to obtain information about male fertility (1). Therefore, morphometric analysis is a method used to evaluate morphology and can be used to determine sperm defects that occur mainly in the head, including macro or microcephaly (2). There are numerous coloring techniques that can be used on domestic animals and humans. Although studies indicate that different staining techniques produce similar results, there are reports of significant differences in staining intensity and contrast, and in the size and shape of sperm (1;3). Therefore, the present study aimed to analyze the influence of the dyes Bromophenol Blue (AB), Eosin-Nigrosine (EO), Silver Nitrate (NP) and quick panoptic (PO) on the head area and total length of spermatozoa from sheep and goats. Ejaculates were obtained from four hairless sheep (24 months of age and 40 kg of body weight) and three goats (36 months of age and 50 kg of body weight). Weekly collections were carried out with the help of a female in natural estrus as a mannequin and an artificial vagina. The ejaculates were diluted in Tris to a final concentration of 100×106 spermatozoa/ml and then smears were made and stained with AB, EO, NP and PO, in addition to an uncut slide as a control. In total, 2,500 sperm cells (500 sperm of each color and control) were digitized and evaluated by ImageJ, software for image processing and analysis. The parameters head area and total sperm length were measured in µm. The data were analyzed using the SAS 9.1 statistical program and subjected to ANOVA. Values were considered statistically significant when ($P<0.05$). In the results found, it was possible to observe that the lowest values for head area were with NP in both species and the highest were with EO (goats) and PO (sheep). For total length, the lowest values were collected in the NP for both species, while the highest values were observed in the control for goats and in the quick panoptic for sheep. It is concluded that the dyes used significantly altered the morphometric configurations of sheep and goat sperm.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

THE IMPACT OF DIET ON THE ONSET OF REPRODUCTIVE BEHAVIORS IN HAIRLESS LAMBS

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The production of sheep is one of the most representative livestock activities in Brazil, especially in the Northeast region, with animals locally adapted to the adverse conditions of the area. However, other factors affect the reproductive performance of these animals, which is determined by genetic merit, environment, management, and nutrition, where nutritional manipulation can affect neural systems, influencing behavior expression (1) (2). In this approach, the objective was to evaluate the reproductive behaviors of F1 Dorper X Santa Inês crossbred lambs subjected to different levels of food restriction, assessing the onset of puberty in relation to diet and age. Thirty prepubertal F1 Dorper x Santa Inês crossbred lambs, with an initial body weight of 31.87 ± 0.5 kg and 157 days of age at the beginning of the experiment, were used. The animals were distributed in a completely randomized design, in a 1 × 3 factorial scheme, being one sex class (30 males) and three food levels (ad libitum, 30%, and 70%). The sheep were kept under adequate sanitary conditions, vaccinated, dewormed, identified, and allocated in individual brick stalls (2 m²) equipped with feeder and drinker. For the analysis of sexual behaviors, visual assessments were performed during the collection period, and the recorded reactions were: Flehmen Reflex, Penile Exposure, Mount, and Ejaculate, as established in previous methodologies (3). All statistical analyses were performed with R (R Development Core Team, 2017). The results obtained suggest that animals kept ad libitum had an increase in parameters related to mounting, exposure, and ejaculation, although no viable sperm cells were found in the ejaculates, suggesting that the animals were not yet pubertal. Animals subjected to food restriction show physiological and behavioral changes in parameters related to reproduction, these results corroborate the findings of a previous study (4). A relationship between the Flehmen reflex and age can also be observed, as at 202 days all animals already expressed this behavior. According to previous research (5), the Flehmen reflex precedes other sexual behaviors, such as mounting, visually signaling the onset of sexual interest in animals. It is concluded that there are no advantages in subjecting rams intended for reproduction to food restriction. However, it was still possible to evaluate whether poor nutrition in the early life stage of lambs will result in future problems for seminal quality and reproductive efficiency, even though it is negative for the onset of reproductive behaviors.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Sperm traits of Rabo Largo rams during the dry season raised in northeastern Brazil

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Rabo Largo sheep have become relevant in northeastern sheep farming, being characterized morphologically by having wool and being susceptible to thermal stress. Thus, this study aimed to evaluate the seminal parameters of Rabo Largo rams raised in confinement during the dry period of the year in Northeast Brazil. With this purpose, three Rabo Largo rams (16 months; 40 kg), vaccinated and dewormed, were placed in a collective pen (98 m²; Department of Animal Science at the Federal University of Ceará, Fortaleza-CE; -3°44'33' '; -38°34'33") and fed Tifton hay and concentrate, with access to water and mineral salt ad libitum (NRC, 2007). These rams underwent semen collections weekly during the months of October and November, totaling eight collections. The semen was collected through an artificial vagina, with the help of a female, so that after collection the volume (mL) was measured and an aliquot was used to evaluate motility (mass motility, total motility and individual sperm motility), sperm morphology (smears stained with bromophenol blue), assessed by bright-field microscopy. Another aliquot was used to evaluate sperm concentration using a spectrophotometer (λ=490 nm). Given the descriptive nature of the study, the data were grouped and the mean and standard error values were obtained. Thus, the rams had a scrotal circumference of 27.7 ± 2.65 and an ejaculate volume of 0.6 ± 0.24 mL. The kinetic parameters of the collected samples showed mass motility of 4.3 ± 0.61, individual sperm motility of 4.6 ± 0.58 and total motility of 77.9 ± 8.84%, with sperm concentration of 2.1 ± 0.83 x 109 sperm/mL. Regarding sperm morphology, a moderate percentage of normal sperm was observed (59.1 ± 0.23%), with emphasis on abnormalities present in the tail (26.0 ± 0.13%), followed by those observed in the head of the sperm (11.9 ± 0.11%) and finally, defects in the intermediate part (3.0 ± 0.02%). Therefore, it can be observed that the animals achieved the desired seminal characteristics only for the variable progressive individual motility (≥ 3.0), being insufficient for sperm normality (≥ 90%) when compared to the values recommended by the Colégio Brasileiro de Zootecnia. This fact may be related, in addition to the time of year, to the age of the animals, as sheep of native breeds reach sexual maturity later. Thus, during the dry season, Rabo Largo rams aged less than 24 months have semen with high sperm motility, but with a moderate number of normal sperm. Therefore, there is a need to improve the environmental conditions inside the sheds so that the rams have greater thermal comfort and sperm parameters and, consequently, better reproductive capacity.

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MALE REPRODUCTIVE BIOLOGY

Conditioned medium from human Wharton's jelly cells maintains the mitochondrial potential of murine spermatozoa

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In spermatozoa, mitochondria are organelles responsible for supplying energy through aerobic metabolism in a process called oxidative phosphorylation. This process enables spermatozoa to acquire motility and enough energy to reach the oocyte and fertilize it. In this sense, mitochondria are fundamental organelles for spermatozoa, and dysfunctions can result in a reduction in fertilization potential. Therefore, using substances that can increase mitochondrial potential is of great value. It is known that conditioned medium from Wharton's jelly mesenchymal stem cells (WJ-CM) contains a series of growth factors and cytokines that can assist in the sperm capacitation process (1). Therefore, the objective of this study was to evaluate the mitochondrial potential of Wistar rats spermatozoa after incubation in WJ-CM. To do this, Wharton's jelly fragments were dissected and cultured in vitro for 72 hours to produce the conditioned medium. Subsequently, spermatozoa from 1 male rat were collected from the epididymal tail and distributed in Eppendorf tubes at a concentration of 2x105 and subsequently, 2 million of these sperm were incubated. The incubation medium consisted of α-MEM supplemented with 1.25 mg of bovine serum albumin, using different proportions of conditioned medium (25, 50, 75, 100%) for a final volume of 1 mL. Incubation was carried out at 37°C in a humidified atmosphere with 5% CO2 for 1 hour. To measure mitochondrial potential after incubation, spermatozoa were stained with 2.0 mM JC-1 and then smeared on a slide, followed by evaluation under a fluorescence microscope. Images of 4 random fields in each group were obtained and fluorescence intensity was quantified using IMAGE J software. Data were expressed as mean ± SD (Standard Deviation). Data normality was performed using the Shapiro-Wilk normality test followed by One-Way ANOVA analysis of variance with Tukey's post hoc using the GraphPad Prism 9.0 software (Graphpad Software, Inc., San Diego, USA). As a result, it was observed that the mitochondrial potential of sperm remained unchanged, with no significant difference between the groups (CF = 0.924±0.11; AL = 0.913±0.06; 25 = 0.933±0.05; 50 = 1.012± 0.09; 75 = 1.006±0.009; 100 = 0.963±0.05). These findings indicate that WJ-CM did not exert cytotoxic effects on spermatozoa, which possibly occurred due to the quantity of beneficial molecules that may be present in the medium. In summary, it can be concluded that although WJ-CM did not directly affect mitochondrial potential, other parameters should be evaluated, such as sperm fertilization capacity after incubation, in order to clarify the impacts that WJ-CM may have in the field of reproductive biotechnologies.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

INFLUENCE OF DIFFERENT DYES ON THE TAIL SIZE OF SPERMATOZOA IN SMALL RUMINANTS

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The assessment of semen quality in breeding males is a crucial step in selecting animals with good fertility characteristics. However, during this process, various factors can interfere, such as the collection method, sample handling, and the use of dyes, which can influence the visualization and morphological characteristics of sperm cells. This study aimed to evaluate the effects of different dyes on the morphometry of the tail of sperm cells in small ruminants. Seven sexually mature animals were used, four sheep and three goats, with average weights of 58kg and 50kg, respectively. The animals were kept in individual pens with access to food and water and were subjected to intensive management. Semen samples were collected weekly using a conditioned female and an artificial vagina and sent to the Laboratory of Animal Reproduction Studies (LERA/UFC) for analysis. Each ejaculate was evaluated for volume (ml) and sperm concentration (×10^9 sptz/ml) using spectrophotometry. Subsequently, the semen was diluted in TRIS to obtain a final concentration of 100×10^6 sptz/ml. Five aliquots of each diluted semen sample were stained with different dyes: Bromophenol Blue (BB), Eosin-Nigrosin (EN), Silver Nitrate (SN), and Rapid Panoptic (RP), while unstained semen smears were used as controls. Images of 500 sperm cells from each dye and the control were digitized and analyzed using ImageJ software to measure the length of the sperm tail in micrometers, totaling 2500 sperm cells analyzed for each species. The data were subjected to analysis of variance (ANOVA), and the means were compared using Tukey's test at a significance level of 5%. The results showed that the different dyes significantly affected the morphometric measurements of sperm tail. In the BB, EN, SN, RP, and control dyes, the following values for flagellum length in goats were obtained: 58.24b, 58.81a, 58.32ab, 57.69c, 58.38ab. Thus, the smallest value for the tail length of goat sperm cells was observed in RP ($P<0.05$). For sheep, it was possible to observe that the highest value was observed in RP (70.49a), while the lowest value was observed in SN (62.42e). However, it is worth noting that the BB dye (63.17d) preserved the morphometric characteristics of the flagellum best, as it was the dye that obtained the value closest to the control (64.48c). The value obtained for this parameter in the EN dye was 66.58b. Therefore, choosing the appropriate dye is fundamental to preserve the morphological characteristics of sperm cells and reduce variation in seminal quality assessment results, not only in small ruminants but also in other studied species.

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MALE REPRODUCTIVE BIOLOGY

Global proteomic analysis of European quail (*Coturnix coturnix coturnix*) testes

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Quails are animals characterized by rapid growth, achieving sexual maturity in a short period (35 to 42 days), and high reproductive rates. Proteomics plays a crucial role in examining and identifying reproduction-related attributes that can affect the reproductive and productive performance of these birds. The objective of this study is to map the proteome of testicular tissue from adult quails. Two pairs of testes from 21-week-old European quail (Coturnix coturnix coturnix) males were used. For protein extraction proteins, 10 mg of lyophilized samples from the testes were resuspended in 500 µl of a lysis solution containing 0.1% Triton X-100, homogenized using a vortex shaker, and kept at 4°C for one hour with gentle agitation every 10 minutes. Subsequently, 400 µl of sample buffer (containing 7 M urea, 2 M thiourea, 40 mM dithiothreitol, and 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) were added and sonicated for one minute. The samples were then centrifuged at 12,000 g for 30 minutes at 4°C, and the supernatant was rese rved for quantification using the Bradford method. After this process, proteins were subjected to trypsin digestion, desalting and label free mass spectrometry analysis (Q Exactive Plus, Thermo Scientific, USA). Raw files were then analyzed using Pattern Lab V (http://www.patternlabforproteomics.org/), and Gallus gallus databank from Uniprot. Protein interactions and functional clusters were assessed by String (https://string-db.org). Our methods allowed the identification of 557 proteins in the quail teste samples. The most abundant proteins were identified as Hypoxia up-regulated protein 1; Sodium/potassium-transporting ATPase subunit alpha-3; 26S proteasome non-ATPase regulatory subunit 1; Serine/threonine kinase 31; Sodium/potassium-transporting ATPase subunit alpha-2; Cytochrome c oxidase subunit 6C; Sodium/potassium-transporting ATPase subunit alpha-1; Histone H4; Histone H4 type VIII; Vesicle-associated membrane protein 3 (Synaptobrevin-3). Major gene ontology classes related to biological processes were defined as cellular process, metabolic process, cellular metabolic process, organic substance metabolic process, among others. Principal cellular components were established as cellular anatomic entity, intracellular anatomical structure, cytoplasm, organelle, and intracellular organelle; as molecular function, as binding, organic cyclic compound binding, heterocyclic compound binding, ion binding, and structural molecule activity. Based on the analysis of KEGG pathways, the proteome of quail testes mainly related to ribosome, carbon metabolism, biosynthesis of amino acids, glycolysis / gluconeogenesis, citrate cycle (TCA cycle), gap junction, phagosome, metabolic pathways, pyruvate metabolism, protein processing in endoplasmic reticulum and Proteasome. Based on WikiPathaway platform, G protein signaling pathways, calcium regulation, mRNA processing were predominantly linked to the quail proteome as well. Thus, our methods allowed the description of a comprehensive overview of the proteome of the gonads from adult quails raised in the Brazilian Northeast. Protein identities and clusters indicate the complex mechanisms played by somatic and germ cells. Proteomics is a valid tool for understanding the molecular mechanisms underlaying testicular function and reproductive biology.

Animal Reproduction

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MALE REPRODUCTIVE BIOLOGY

Casein extracted from milk: cryoprotective base for the formulation of a sanitary obstacle-free seminal extender and effective in sperm preservation through cold

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Cryopreservation of semen is a reproductive biotechnology that is gaining increasing prominence (1), with skim milk-based semen extender being one of the most commonly used for this purpose (2). This is because casein, in particular, acts as a non-penetrating cryoprotectant (3). However, due to its excellent nutritional medium, it facilitates microbial growth, consequently raising sanitary risks. Thus, the addition of antimicrobials is crucial (4), albeit predisposing to the emergence of more resistant microorganisms (5). Seeking to overcome existing sanitary challenges and promote microbial resistance, this study aimed to develop a chemically defined extender, with isolated milk casein as its central base, and to assess its cryoprotective potential on refrigerated ovine spermatozoa. In this regard, three ejaculates from three sexually mature and fertile ovine breeders, after evaluation and approval, were individually fractionated and diluted according to experimental groups [G1: 2.5g casein/L of Tris-buffer solution; G2: 0.5g casein/L of Tris-buffer solution (3.605g Tris-hydroxymethylaminomethane, 2.024g citric acid, 1.488g fructose, and 100mL Milli-Q water, pH 6.8); G3: 0.25g casein/L of Tris-buffer solution; G4: egg yolk-Tris extender (67.2mL Tris-buffer solution, 20.0mL egg yolk, and 12.8mL Milli-Q water. Control group)], to a concentration of 80 x 106 spermatozoa/mL. Subsequently, the samples were refrigerated (5°C) and evaluated at post-dilution moments (37°C), as well as at zero, 24, and 72 hours upon reaching 5°C, for plasma membrane integrity (PMI), using the double staining technique with carboxyfluorescein diacetate and propidium iodide fluorochromes (6). A total of 200 cells were analyzed under epifluorescence microscope (400x) and classified as intact when stained green or damaged when stained red. Data were expressed as means and standard deviations (mean±SD) and analyzed, after arcsine transformation, by one-way ANOVA, followed by Tukey-Kramer multiple comparison test, with a significance level of 5%. No significant differences (P>0.05) were found for PMI among experimental groups, regardless of the analysis time (G1: 37ºC=66.00±25.23; 0h=18.50±28.57; 24h=67.50±10.05; 72h=34.00±23.15. G2: 37ºC=57.00±17.76; 0h=26.00±8.84; 24h=43.50±15.69; 72h=51.00±24.68. G3: 37ºC=63.00±23.64; 0h=59.00±20.25; 24h=57.50±16.89; 72h=44.00±16.65. G4: 37ºC=72.50±12.62; 0h=62.50±16.06; 24h=67.50±1.15; 72h=72.50±26.15). Casein is attributed to sequestering seminal plasma proteins, thereby preventing cellular membrane injuries (7), which supports the described findings. It is concluded that isolated milk casein is a key constituent in the formulation of a chemically defined extender, as it maintains the viability of cryopreserved ovine spermatozoa.

Keywords: goat; seminal diluente; sperm integrity; semen refrigeration.

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Animal Reproduction

X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Agenesis of the penile bone in dogs: case report

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The penile bone consists of an elongated structure that lies almost completely within the glans and has a ventral groove that houses the penile urethra. In dogs, the penile bone facilitates penetration at the time of intercourse. Penile bone agenesis is a rare abnormality of the genital system, characterized by the non-development of the penile bone. The objective of this study is to describe a case of penile agenesis in a young dog. A male dog, 1 year and 4 months old, neutered, of the Shih-Tzu breed was admitted to the Unileão Veterinary Hospital with a history of painful urination, drip and constant licking of the penile region. The owner reports that after copulation with the mother, the animal had a very swollen penile region and that the animal injured the region near the scrotum, creating a hole through which the urine came out. During clinical evaluation, the presence of fistulas in the foreskin region, apathy, hyperthermia, pain and discomfort, with inflammation of the penile region and hematuria was observed. Ultrasonography and abdominal radiography were requested, in addition to laboratory tests such as complete blood count, urea and creatinine levels. On ultrasonography, it was possible to see that the testicles were symmetrical and in the usual topography, without alterations, and the prostate was preserved for the patient's age and size, with normal contours, echogenicity and echotextures. The bladder was distended with anecogen content and an enlarged wall thickness (0.45 cm) and irregular appearance, which was compatible with acute cystitis. The other abdominal organs appeared within normal range at the time of the examination. For the radiographic examination, ventrodorsal and laterolateral projections were performed. In this study, it was possible to observe that the patient did not have a penile bone, characterizing a case of agenesis. The patient was referred for a surgical procedure, where scrotal urethrostomy was performed. After accessing the urethra, a urethral probe No. 10 was placed to empty the organ. Subsequently, the urethra was anastomosed to the skin with a 4-0 nylon suture in a simple pattern separated into the urethral mucosa. To the best of our knowledge, this is the first case report of a dog with penile bone agenesis. In dogs, the routine of cases with penile bone is mainly related to fractures, which are associated with urinary tract obstruction or laceration of the urethra. In more severe cases, it may be necessary to perform penis amputation. In the present case, after urethroplasty, the animal was able to urinate normally.

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MALE REPRODUCTIVE BIOLOGY

Correlation of seminal quality parameters evaluated by flow cytometry in young Alpine bucks semen

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Reducing feed cost without compromising reproduction performance is essential for a successful livestock operation. Moreover, is fundamental to evaluate the feed impact on the seminal quality. Thus, post-cryopreserved seminal quality was compared by flow cytometry form young Alpine breed bucks fed maintenance (n=5) or ad libitum (n=5) diets. The diets were formulated according to AFRC (1998), composed by alfalfa hay, ground corn, and soybean meal. Animals had ad libitum access to water and mineral mix. Ten young bucks between seven to eight months of age were randomly housed in individual pens, over a 35-d experimental period. Ten ejaculates were collected using an estrus female and an artificial vagina at 37 °C. Progressive sperm motility and vigor were evaluated as recommended by the Brazilian College of Animal Reproduction (1). Seminal analysis and frozen protocol were procedure as Penitente-Filho et al. (2). Sperm quality analyses were performed using a FACSVerse™ cytometer (BD Biosciences, San Diego, CA, USA) as described by Al-Kass et al. (3). It was evaluated the intracellular peroxide concentrations (reactive species of oxygen - ROS), staining with 2',7' –dichlorofluorescein diacetate (DCFDA; 1 mg/mL); integrities of acrosomes and plasma membranes using fluorescein isothiocyanate - Pisum sativum (FITC-PSA; 0.1 mg/mL) and Propidium Iodate (0.5 mg/mL); and lipid peroxidation in sperm plasma membranes using the C11-BODIPY compound Y (1 mg/mL). Statistical analyses were performed using Sigma Plot 27.0 (Systat Software Inc., San Jose, CA, USA). The sperm volume was greater in ad libitum treatment ($p<0.05$). Gross motility, sperm motility, sperm vigor and sperm concentration did not differ between treatments ($p<0.05$). The percentage of spermatozoa without intracellular ROS was lower in maintenance treatment. For the maintenance treatment, membrane integrity was correlated with acrosome integrity ($r=0.550$; $p=0.0001$), and with absence of lipid peroxidation ($r=0.596$; $p=0.0001$). Additionally, acrosome integrity and lipid peroxidation were correlated ($r=0.592$; $p=0.0001$), as well as absence of ROS and absence of peroxidation ($r=0.348$; $p=0.032$). On the other hand, ad libitum treatment, membrane integrity was correlated with acrosome integrity ($r=0.370$; $p=0.016$), absence of ROS ($r=0.347$; $p=0.024$) and absence of lipid peroxidation ($r=0.597$; $p=0.0001$). Moreover, acrosome integrity and lipid peroxidation were also correlated ($r=0.709$; $p=0.0001$). Feeding young Alpine bucks maintenance diet did not compromise the percentage of plasmatic and acrosomal membrane integrity, as well as the occurrence of membrane peroxidation. The accumulation of intracellular ROS leads to lipid peroxidation due to the formation of stable products such as malondialdehyde (MDA), one of the main agents that deplete seminal quality (4). Nevertheless, the correlation of the maintenance treatment indicates that sperm population with whole membrane it is associated with the sperm population without acrosomal and lipid peroxidation damage, even though maintenance had lower sperm percentage of intracellular ROS. Overall, despite of the reduction of sperm volume and sperm without ROS percentage, young Alpine bucks fed maintenance had similar semen quality, once neither physical nor chemical parameters did not differ (5).

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Animal Reproduction

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MALE REPRODUCTIVE BIOLOGY

HISTOLOGY AS A TOOL FOR EVALUATING GONADAL MATURITY IN BOTTLENOSE DOLPHINS (Tursiops truncatus)

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The bottlenose dolphin (Tursiops truncatus) is a widely studied and distributed species, yet its inclusion in the IUCN Red List of Threatened Species reveals the challenges it faces (1). Males of this species employ various reproductive strategies, although the characteristics of their testicles remain poorly understood (2). Therefore, the present study aimed to classify sexual maturity through histological analysis of a T. truncatus specimen. A male specimen stranded in the Potiguar Basin-RN in 2014, recorded during beach monitoring by the Cetacean Project of the Costa Branca (PCCB-UERN) team, was utilized. The carcass was forwarded to the Marine Biota Monitoring Laboratory (UERN). During necropsy, the testicles were collected, weighed, measured, and photographed. Sexual maturity classification was performed according to Rosas and Monteiro-Filho (2002) (3). Macroscopically, the testicles were enveloped by a smooth, grayish, thick, and resilient capsule, known as the tunica albuginea, which surrounded a dense superficial vascular network. The testicular cut surface revealed septa extending from the albuginea towards the medullary portion of the organ, creating intercommunicating lobules that fused to form a faint mediastinum, located centrally. Microscopically, the testicles exhibited Leydig cells distributed in abundant interstitial tissue between the closed seminiferous tubules. On the other hand, the seminiferous tubules were characterized by irregular tubular formations, lined with epithelium containing numerous spermatogonia, Sertoli cells, a second layer of primary spermatocytes, and some tubules containing secondary spermatocytes, emerging from the deep region of the testicular parenchyma towards the central portion. The presence of Leydig interstitial cells, occasionally exhibiting cytoplasmic vacuoles and distributed singly or in small groups in the connective tissue, sharing space with blood vessels and lymphatics, was also observed. The specimen was classified as prepubertal immature, due to the presence of seminiferous tubules with absence of a lumen, and presence of spermatogonia and primary spermatocytes. The characteristics of Leydig cells observed in this study are similar to the intertubular distribution pattern of testicles in rams, bulls, and primates (4). Thus, these findings contribute to the advancement of scientific knowledge and may facilitate the development of reproduction techniques to classify maturity status and thus aid in species conservation.

Keywords: Sexual determination, Cetacea, Reproduction, Delphinidae.

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