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Morphological characterization of brown brocket deer (*Mazama gouazoubira*) sperm ultrastructure by transmission electron microscopy

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The present study was conducted to describe the ultrastructure of brown brocket deer (*Mazama gouazoubira*) sperm using transmission electron microscopy (TEM). The study was approved by Ceara State University Ethics Committee (number 7913746-2017) and by the System of Authorization and Information on Biodiversity (number 60925). Before the procedures, animals were sedated with 5 to 10 mg/kg ketamine hydrochloride and 0.5 to 1.5 mg/kg xylazine hydrochloride, both IM (Cursino; Duarte, Royal Society Open Science, 3:1-9, 2016). Two adult animals (2-5 years old) had their semen collected using electroejaculation (n=2 ejaculates). Subjective progressive motility, vigor and viability (using eosin-nigrosin) were immediately analyzed, and an 80 µl aliquot from each animal was separately processed for TEM analysis. The samples were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in phosphate buffer (0.2M; pH 7.4). Three phosphate buffered solution (PBS) washes were performed, followed by post-fixation in osmium tetroxide for 1h and three PBS washes. Then, samples were dehydrated in series of acetone (50%, 70%, 90% and three times in 100%), and embedded in Epon resin. Ultrathin sections (50 nm) were manually stained with uranyl acetate and lead citrate. For both animals, 25 different fields were randomly selected, evaluated by TEM and photographed for later analysis. Progressive motility was 80% and vigor 5 for both collections. Viability was 87% and 90.5% for each animal. In TEM, the head and the flagellum (tail) were tightly surrounded by a plasma membrane (PM). The head contained a nucleus (NU), enveloped by the nuclear membrane, the acrosome (AC) and the sub-acrosomal space (SAS). The NU was electron-dense with smaller electron-lucent areas. The AC was enclosed by inner and outer membranes, and it extended beyond the apex of the head until the equatorial segment where it narrowed caudally. The caudal portion of the NU was covered with a post-acrosomal sheath. The implantation fossa limited the nuclear posterior end and led to the connecting piece which was formed with the capitulum, the proximal centriole and the segmented columns (SC), structures mainly responsible to attach head to tail. The SC ended as the outer dense fibers appeared (ODF) in the midpiece (MP) anterior region, surrounded by the mitochondrial sheath and PM. Enclosed by ODF, there was the axoneme (AX) characterized by nine doublets of microtubules forming a cylindrical bundle arranged around a pair of microtubules. In longitudinal view, 60 mitochondrial spirals were counted in this species. In the principal piece, the AX and ODF were encircled with a fibrous sheath (FS) and PM. Fibers 1,5 and 6 were larger than the others. The FS was attached to the central pair of microtubules in fibers 3 and 8. Characterizing the ultrastructure of *M. gouazoubira* sperm may help not only in sperm morphological studies, but also, in comparative biology studies.

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Evaluation of testicular thermoregulation by infrared thermography and its influence on seminal quality in Nellore bulls

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Testicular temperature should be below body temperature for normal spermatogenesis. An increase in testicular temperature, even if moderate, can cause a drastic decrease in sperm production and seminal quality, leading to a reduction in the bull fertility. Infrared thermography (IRT) can provide important information about the bull's ability to maintain testicular temperature, since the superficial temperature of the scrotum is closely related to the internal temperature of the testicle. Therefore, the objective of the study was to evaluate the relationship between testicular temperature assessed by thermographic images and the seminal quality of Nellore bulls. The experiment was conducted at Instituto de Zootecnia, Sertãozinho-SP. Forty-five semen samples were obtained by 15 Nellore bulls (n=3 ejaculates/bull) with 30 days interval between collections. The surface temperatures of the scrotum were measured by IRT (T300 FLIR Systems®) using two lines, traced across the scrotum image to measure the minimum and maximum proximal (TPMI and TPMX) and minimum and maximum distal (TDMI and TDMX), respectively. Minimum and maximum images of the ocular globe (GBMI and GBMX) and the lacrimal caruncle (CARMI and CARMX), both on the left side of the animals' head were evaluated to verify the thermal state of the bull. Semen samples were collected with electroejaculator. After each collection the sperm kinetics of fresh semen was determined by Computer-Assisted Sperm Analysis (CASA, IVOS-14, Hamilton Thorne Bioscience®, USA) and the following parameters were analyzed: total motility (MT,%), progressive motility (MP,%) and percentage of rapid sperm (RAP,%). For analysis of sperm morphology, aliquos of each semen sample was diluted in 4% saline formalin solution and evaluated by differential interference contrast (DIC) microscopy with 100X. Two hundred cells were counted per sample and the abnormalities classified as major (DEFMA), minor (DEFMI) and total (DEFTO) defects. Seminal parameters (dependent variables) were analyzed with a multiple regression model including all skin surface temperatures as predictor variables. The variation of MT (R^2 = 0.26), RAP (R^2 = 0.28) and DEFMI (R^2 = 0.26) was partial explained by temperature measured at the distal poles of the testis. The temperature of TDMX (32.9±2.2ºC) was negatively related to MT (P=0.023) and to RAP (P<0.001), however CARMI (33.9±2.2ºC) and TPMI (31.4±2,5ºC) were positively related to MT (P<0.001) and to RAP (P=0.064), respectively. TDMI (31.4±2,5ºC, P=0.021) and GBMI (33.4±1.6ºC, P=0.028) were positively related to DEFMI. The results suggest that the temperatures of the distal pole of testis obtained by IRT explain part of the variation of total motility, percentage of rapid sperm, and minor defects of spermatozoa of Nellore bulls. Acknowledgment: FAPESP (process 2014/11.304-3 and 2015/24.174-3) and Botupharma®, Botucatu, São Paulo, Brazil.

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Use of low-density lipoproteins and chicken egg yolk as non-penetrating cryoprotectors for cryopreservation of Peruvian Paso Horse semen

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To improve cryopreservation efficiency for equine semen, it is important to use new diluents that contain non-penetrating cryoprotective agents. The principal objective of this study was to evaluate semen quality in Peruvian Paso Horse cryopreserved with low density lipoproteins (LDL) and chicken egg yolk. Semen was collected from 10 horses using an artificial vagina, and macroscopically evaluated to determine volume, color, appearance and pH. Semen was diluted with EquiPlus® at 37° C, and then sent to the laboratory to be cryopreserved using the protocol described by Castro et al. (Veterinary Sciences, 2016: 45-64). In the lab, each sample was divided into two aliquots and either 3 gr of LDL or 3 gr of egg yolk were added to each aliquot. The technique developed to obtain LDL was based on the methodology proposed by Gonzales (Gonzales, R., Extraction of low density lipoproteins of egg yolk [Internet, Downloaded May 2019]). After cryopreservation, samples were thawed and microscopically evaluated for motility, viability and morphology, as determined by Eosin Nigrosine for viability, Endosmosis test (HOS) for membrane's functionality, and Bengal Rose for malformations. These tests were based on the standard operating procedures (SOPs, 2018), in the Animal Biotechnology Laboratory, Universidad Católica de Santa Maria. Data were analyzed by central tendency and dispersion statistics. The significance was determined by Student's t-test for independent data at level of $\alpha = 0.05$. The average of macroscopic parameters observed was: volume: 41.5 ml; color: milky white appearance; and pH: 7.46. These parameters were not statistically different ($p > 0.05$) between stallions. Better seminal quality was observed in straws cryopreserved with LDL compared to egg yolk, with statistical differences ($p < 0.05$) for the variables: motility at thawing (48.9% vs. 27.5%), motility at 20 min post thawing (38.2% vs. 19.3%); progressive motility (38.3% vs. 21.5%); viability (45.9% vs. 31.7%); and functional membrane (59.3% vs. 41.6%) for LDL and yolk, respectively. There was no statistical difference for sperm morphology ($p > 0.05$) between groups. In conclusion, the use of LDL improved cryopreservation of Peruvian Paso Horse semen compared to egg yolk. This is likely because of the isolation of the LDL as pure lipoproteins compared to yolk that contains other components.

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Stallion sperm capacitation with exogenous modulators: Effect on the conventional *in vitro* fertilization of equine oocytes

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A reliable protocol for equine IVF has not yet been established. The aim of the present study was to evaluate sperm capacitation conditions with exogenous cAMP-pathway modulators (dbAMPc and IBMX) and a cholesterol remover (MβCD) on sperm capacitation and assess the functionality of these conditions in a heterologous zona pellucida (ZP)-binding assay and in IVF with *in vitro* matured mare oocytes. Fresh semen was collected from 3 stallions, diluted to $10x10^6$ sperm/mL in non-capacitating and capacitating conditions with inductors and incubated for 0 and 4h at 38°C in air atmosphere using Tyrode´s medium. Membrane fluidity (MC540+), Ca^{2+} levels (FLUO 3-AM), tyrosine phosphorylation (PY mAb) and acrosomal exocytosis (PNA/FITC) were assessed by flow cytometry. Bovine and equine ovaries were collected from local slaughterhouses. In ZP-binding assay, cumulus-oocyte complexes (COCs) were aspirated, selected and denuded from granulosa cells and co-incubated in capacitated sperm for 1 hour and ZP-bound spermatozoa were counted under an epifluorescence microscope. For IVF, collected mare COCs were matured in DMEM-F12 supplemented with FSH and 10%FBS for 32 h at 38.5°C, 5% CO2% and saturation humidity. COCs were co-incubated with capacitated sperm for 18 h and presumptive zygotes were cultured in DMEM-F12 + 10%FBS for 7 days in low O_2 tension. Data are shown as means ± SD of 3 replicates for each stallion. ANOVA was used after arcsine transformation of the proportional data and Tukey's post-test to find difference between groups. Significance difference were considered if $P<0.05$. The results obtained confirmed significantly higher ($P<0.05$) tyrosine phosphorylation, $Ca^{2+}i$, membrane fluidity and acrosomal exocytosis (3300±210AU [arbitrary units], 4493±887AU, 90±5% and 50±6%, respectively) in Tyrode medium containing inductors of capacitation compared to the non-capacitating conditions (1400±40AU, 2615±373AU, 10±4% and 11±2%, respectively). ZP-binding assay revealed higher number of stallion sperm ($P<0.05$) attached to the ZP of bovine oocytes (410) compared to non-capacitating conditions (105). IVF experiments performed only in capacitating conditions showed an average of 31% of cleavage 3 days after insemination and embryos cleaved to the 8 to 16-cell stage. Despite assessing embryonic development until day 7, none of the embryos developed beyond this point. In conclusion, stallion sperm incubated with exogenous inductors of capacitation show cellular and molecular changes consistent with sperm capacitation. Furthermore, ZP and IVF trials confirm the functionality of stallion sperm incubated under these conditions. Acknowledgement: Funding support from CONICYT, Chile grant FONDECYT 1160467, and provision of ovaries from Frigorifico Temuco and Nueva Imperial.

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High intensity interval training improves the sperm motility of spontaneously hypertensive rats

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Hypertension is a cause of low fertility in men because it reduces testosterone production and spermatogenesis. Exercises are indicated to decrease blood pressure and improve overall health. The objective of this study is to verify the sperm motility of spontaneously hypertensive rats (SHR) submitted to high intensity interval training (HIIT). For this, Wistar-Kyoto rats, male, adult, with and without spontaneous hypertension were distributed in 3 groups: K-G (control of Wistar-Kyoto rats without hypertension submitted to HIIT, n=5); SHR-G (group of SHR rats, n=9); and SHR-HIIT-G (group of SHR rats submitted to HIIT, n=9). The treadmill HIIT training was realized for 5 days/week for 8 weeks, for 50 minutes approximately, based on the maximum exhaust speed, with active rest intervals according to the protocol of Haram et al. (Cardiovascular Research, 81: 723-732, 2008). The animals initially underwent an adaptation to the HIIT training, which consisted of running on the treadmill for 2 minutes at 0.5 km / h, followed by 5 minutes of rest and soon after 3 minutes at the speed of 0.7km / h, increasing 0.2 km / ha every 3 minutes until it reached 1 mmol / LL above the initial test. After the adaptation period HIIT was performed with 5 minutes of heating at 40% of the lactation threshold. After warming up, the training was started with 3 minutes at 60% of the lactate threshold followed by 4 minutes interval of 85% of the lactate threshold, which was repeated seven times each session. The vas deferens with the sperm were collected. The evaluation of sperm motility was performed immediately at the time of euthanasia. The motility was classified by visual observation under a microscope in: mobile with progressive trajectory; mobile without progression; and motionless. The results were analyzed by analysis of variance (ANOVA), followed by the Tukey test ($P<0.05$). The SHR-G (67.22±1.49) presented lower progressive motility ($P<0.05$) than the K-G (73.10±1.88) and SHR-HIIT-G (73.00±1.11). It is concluded that HIIT improves the sperm motility of SHR rats. Financial support by FAPESP (process number: 2018/22682-0) and PIBIC-EM (CNPq).

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Antioxidant effect of caffeine addition to post-thawed equine semen

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The main objective of this work was to evaluate the antioxidant effect of adding 5mM of caffeine to post-thawed equine semen. One ejaculate of nine stallions was frozen with INRA 82 freezing extender. The straws were thawed performing two treatments: T1) INRA 82, control (without caffeine addition) and T2) T1+5mM caffeine.The antioxidant effect was evaluated after incubation (37 °C, 20min) by nitrite measurement (NO2-), which is an indirect way to investigate the nitric oxide formation (Green et al., Analytical biochemistry, 126: 131-138, 1982) and by hydrogen peroxide concentration (µM / µg protein) by the FOX 2-modified method (Nourooz-Zadeh et al., Analytical Biochemistry, 220: 403-409, 1994). The mean, standard deviation, standard error and variance analysis were performed according to Sampaio (Sampaio, Estatística Aplicada à Experimentação Animal, 17: 189-207, 2002). The NO_2^- and H_2O_2 concentrations presented normal distribution and the mean values were compared by the paired T-test. The significance of $P<0.05$ was considered statistically different. In the present study, lower NO_2^- concentration was observed after the addition of 5mM caffeine to the post-thawed equine semen compared to the control (11.4 ± 2.1^b *vs* 12.8 ± 2.9^a µM/µg of protein, $P<0,05$). However, the H_2O_2 concentration did not differ between the control and the caffeine treated group ($P>0.05$, 36.4±3.6 and 37.0±2.1 µM/µg of protein). Thus, besides increasing stallion sperm motility of thawed semen (Lagares et al., Reproduction, Fertility and Development, 31:142-143, 2019), 5mM caffeine has antioxidant effect, which can be an alternative to increase the fertility rate of post-thawed equine semen.

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L-carnitine effects on the intracellular calcium ion concentration and membrane integrity of post-thawed equine sperm

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The aim of this work was to evaluate the addition of L-carnitine (L-C) to the frozen-thawed semen of equine to improve the sperm integrity and metabolism by measuring the intensity of propidium iodide (PI) and the $[Ca_2^+]$i after different periods of time. Semen from five stallions was collected and frozen with INRA 82 extender. After thawing, the samples were distributed according to the treatments: T1) Control, INRA 82 extender (no L-C addition), T2) T1+0.5mM L-C, T3) T1+1mM L-C, and T4) T1+2mM L-C. The samples were analyzed immediately after L-C addition (t0), and post-, 20 (t20) and 40min (t40). An epifluorescent microscope was used to evaluate the $[Ca_2^+]$i with the Alexa Fluo-4AM, and the propidium iodide (PI) dye to evaluate the sperm membrane integrity. After thawing the semen samples were centrifuged, and the pellet was resuspended with a PBS solution with 6 mM Glucose and Fluo-4AM (20 µmol/L), and incubated at room temperature for 30 min. After 20min incubation, the PI was added to the sample and incubated to the last 10 min. It was used an argon laser to excite the Fluo-4AM (488 nm) and emission of 510 nm, with an epifluorescent microscope (400X). The images were captured each 500 msec. and 600 sec. at 400 Hz, 512x512 pixels. The program ImageJ (Wayne Rasband, National Institutes of Health, USA) was used to analyze the sperm cells images. A linear mixed model was adjusted considering Fluo-4 AM and PI fluorescent intensity of sperm as variables according to the treatments and time evaluated. The stallion was considered as random variable. The Tukey test was used to compare the mean values and a significant level of 5% was considered significant. No significant differences until 40 min incubation among the PI intensity of sperm in 1 e 2 mM L-C treatments were observed (54.0 and 50.4 AU), respectively ($P>0.05$). However, at 20 min incubation there was an increase of non-intact sperm membrane in the control (51.5 to 54.7 UA) and with 0.5 mM L-C (52.5 to 56.4 AU, $P<0.05$). In all time evaluated the 2 mM L-C treatment showed lower PI intensity sperm compared to the other treatments ($P<0.05$). Just the 2mM L-C showed a decrease of $[Ca^{++}]$i intensity during 40min incubation (49.4 to 44.6 AU, $P<0.05$). On the other hand, with 1 mM L-C an increase of $[Ca^{++}]$i intensity until 40min was observed (47.4 to 60.6 AU, $P<0.05$). However, it was not associated with an increase of the PI intensity sperm in this treatment. Thus, the addition of 2 mM L-C to the thawed sperm of equine showed higher protection of sperm membrane integrity, while 1 mM was beneficial to sperm metabolism increasing $[Ca^{++}]$i intensity. In conclusion, both 1- and 2-mM L-C improved essentials sperm characteristics, which play an important role to sperm fertilization capability.

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Efficiency of sexed semen in the *in vitro* fertilization of oocytes obtained from prepubertal heifers

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The increase investigation for the reduction of the time interval between generations is of great interest in dairy farming. However, lower oocyte competence is well-established for prepubertal heifers. The objective of the present study was to compare the use of sexed semen with conventional semen in prepubertal Holstein calves from 7 to 9 months of age (n = 151), related to the number of embryos produced from oocytes recovered by OPU, with a total of 397 (2.63 ± 0.134 per calf) aspirations. The aspirated oocytes were matured for 24 hours, fertilized with sexed or conventional semen of 60 different bulls with proven fertility and standardized performance in the in vitro production of embryos, and cultured in vitro. Means were compared by analisys of variance and Tukey test with a significance of $P \leq 0.05$. The age of the donors when compared to the average number of embryos did not present difference ($P = 0.803$). There was no difference in the type of semen used and number of embryos produced (sexed semen: n = 156, 1.7 ± 0.25 / conventional semen: n = 241, 2.2 ± 0.19 [$P = 0.091$]). We highlight the optimization of the reproductive techniques in younger animals, especially of the dairy herd, since the use of sexed semen was as efficient as conventional semen.