



A001 Physiology of Reproduction in Male and Semen Technology

Morphological changes in sperm cells of stallions of the nordestine breed after thawing

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Keywords: equine, major defects, minor defects.

In spite of its importance for the Northeast region of Brazil, the Nordestino breed is reduced to a few animals, making studies on seminal characteristics, response to cryopreservation and post-thaw fertility of sperm necessary for breed preservation programs. This study evaluated the post-thaw sperm morphology in Northeast breed stallions to estimate fertility and feasibility for commercialization. This study was approved by Ethics Committee in humans and Experimental Animals (CEDEP) of UNIVASF (Protocol number 0006/161012). Were evaluated 19 ejaculates from two stallions (N = 10 and N = 9 ejaculates, respectively), clinically healthy, fertile and with an average of 9.5 years. Two days after the depletion of the extragonadal sperm reserves the ejaculates were collected, diluted (1:1) with Botu-Semen® (Biotech Botucatu, Botucatu, SP, Brazil), kept at 37°C and sent to the protected CPSENS protected from the light, where they were centrifuged in conical tubes (50 mL) at 2200 rpm for 15 min. The supernatants were discarded and the pellets resuspended in Botu-crio® (Biotech Botucatu, Botucatu, SP, Brazil) to the concentration of 106 sperm per mL and evaluated for total motility (TM) and progressive (PM) using CASA. Every ejaculate presented >70.0% of TM and was frozen by the traditional method and stored at -196°C. After thawing in a water bath at 37°C for thirty seconds, a rate of 10 µL of semen was added to 1.0 mL formalin-buffered saline, heated to 37°C, and stored at room temperature for morphology analysis by the method of wet chamber. One drop of this mixture was placed between slide and coverslip and 200 cells were evaluated in 1000x magnification under phase contrast microscope. The total sperm defects were divided into major and minor defects (CBRA 2013). The average percentage of total defects was 5.86±4.22%, where the average of the defects was 2.12±1.93% of major and 3.74±2.29% of minor defects. The major defects were divided into: strongly coiled tail (4.14±2.31%), proximal cytoplasmic droplet (2.58±1.63%), head isolated (1.69±1.49%), incomplete acrosome (1.31±1.19%) and coiled tail (0.89±0.82%). The minor defects were simple coiled tail (5.14±1.93%), thin head (4.58±2.09%), elongated head (3.86±1.78%) and distal cytoplasmic droplets (1,39±1,34%). The results indicate that the analyzed ejaculates of these stallion of the Nordestine breed exhibits excellent morphological quality.

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A002 Physiology of Reproduction in Male and Semen Technology

Evaluation of integrity of acrosomal and plasmatic membranes and the mitochondrial potential of cryopreserved bovine sperm with or without the presence of seminal plasma

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Keywords: centrifugation, filtration, Nellore.

The post-cryopreservation sperm survival rates are not good for most species. In bovine, on average, around half of all the spermatozooids are damaged or destroyed by the freezing, limiting the total efficiency of the semen preservation. As a result, new strategies for cryopreservation are necessary to increase the number of living sperms and the quality of these ones after thawing them. One of the most used strategies for several species is the removal of seminal plasma before cryopreservation, which can be done through centrifugation or filtering of semen. Thus, the aim of this study is to evaluate the integrity of the acrosomal and plasmatic membrane and the mitochondrial potential of cryopreserved spermatozooids in 38 Nelore bulls with or without the presence of seminal plasma. After the semen was obtained through electroejaculation, it was fractioned in three equal aliquot and then the treatments were performed according to Campanholi et al. (VII Congresso Interinstitucional de Iniciação Científica (CIIC), Campinas, São Paulo, Brasil, 2013, p.1-8). Treatment T1 (traditional) consisted of the dilution of semen for the final concentration of 60×10^6 spermatozooids/mL with dilutor BotuBov® (BotuPharma®, Botucatu, Brasil). Treatment T2 (centrifugated) involved a 10-minute centrifugation at 600xg (2200 rpm) to remove the seminal plasma. Treatment T3 (filtered) consisted of filtering the semen through Sperm Filter® (BotuPharma®, Botucatu, Brasil). After centrifugating and filtering the semen, the spermatozooids were resuspended with dilutor BotuBov® in the same concentration as T1. After all the treatments had been performed, the semen was envased at room temperature in pallets (0.5 mL) and frozen using TK 4000® (TK®, Uberaba, Brasil). The propidium iodide probe (Sigma®), FITC-PSA (Sigma®) and Hoescht 33342 (Sigma®) were used to assess the integrity of the acrosomal and plasmatic membrane whereas the probe JC-1 (Molecular Probes®) and H33342 were used to assess the mitochondrial potential. The analyses were performed through flow cytometry using BD® LSR II (Becton Dickinson, Mountain View, CA, USA) and the data were assessed through the program BD FACSDiva™ Software v6.1. The statistical analyses were performed using PROC GLM of SAS. A significance level of 5% of probability has been considered. T2 and T3 have shown a higher percentage of cells with damaged acrosomal and plasmatic membranes (T1=30.61±0.98, T2=38.46±0.98 and T3=39.34±0.98; P<0.05). There was no difference between treatments concerning the percentage of spermatozoa with high (T1=24.28±1.68, T2=28.22±1.68) and low (T1=33.32±2.06, T2=34.03±2.06 and T3=33.70±2.06; P=0.97) mitochondrial potential. Therefore, it has been concluded that the removal of seminal plasma has increased the percentage of damage in plasmatic and acrosomal membranes.

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A003 Physiology of Reproduction in Male and Semen Technology

Cooling systems evaluation in sperm kinetics and integrity of plasma and acrosomal membrane of bovine semen

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Keywords: CASA, Nellore, sperm quality.

One of the main steps in semen cryopreservation process is the cooling curve. The cooling rate may decrease the thermal shock and the damage to sperm cells, and the best results can be obtained using slower and homogeneous cooling curves. In this context, the objective was to compare simultaneously different bovine semen cooling systems and evaluate their effects on sperm quality. Thirty-eight ejaculates from 12 Nellore bulls (3.02±0.5 years of age, 631 ± 119 kg of body weight, and 33.8 ± 1.89 cm of scrotal circumference), collected by electroejaculator, were analyzed. The samples were diluted in the means of one fraction BotuBov® (Botupharma®, Botucatu, Brazil) to final concentration of 60x10⁶ spz / ml. After dilution, they were loaded into 0.5 mL straws and cooled for 5 hours at 5 different cooling systems: System 1 [S1 cooling curve 0.25°C / minute to freeze the machine TK 4000® (Tetakon®, Uberaba, Brazil)]; System 2 [S2, cooling curve of 0.5°C / minute to freeze the machine TK 4000®]; System 3 [S3, refrigerator Minitube® (Minitüb®, Tiefenbach, Germany)]; System 4 [S4, BotuTainer® (Botupharma®, Botucatu, Brazil)], and System 5 [S5, Common household refrigerator]. The sperm kinetics was carried out by CASA (computer analysis of semen System - IVOS® version 14) in two stages: in the diluted semen (without glycerol) before refrigeration (S0) and after 5 hours of cooling in the 5 cooling systems. The variables evaluated were: motility (MOT), progressive motility (PROG) and rapid (RAP). For the evaluation of the plasma and acrosomal membrane integrity, an association of fluorescent probes was used: Propidium Iodide and carboxifluoresceína 6-diacetate (Sigma®, St. Louis, USA). Data were submitted to analysis of variance by proc MIXED (SAS Inst., Cary, USA), and the significance was declared when P <0.05. There was a difference of 5 cooling systems in sperm kinetics and integrity of plasma membrane and acrosome compared with semen evaluated before refrigeration (S0) (P <0.001), but no difference was observed between treatments after cooling. For MOT, the results obtained from each treatment were: S0 = 88.2; S1 = 82.0; S2 = 81.1; S3 = 78.6; S4 = 79.8; and S5 = 78.7 (± 2.60). For PROG: S0 = 70.7; S1 = 54.8; S2 = 56.9; S3 = 56.9; S4 = 57.0; and S5 = 57.2 (± 3.48). For RAP: S0 = 84.6; S1 = 79.4; S2 = 78.9; S3 = 75.7; S4 = 77.2; and S5 = 76.0 (± 2.64). For the lesion of plasma and acrosomal membrane, the results were: S0 = 46.2%; S1 = 52.9%; S2 = 53.5%; S3 = 55.3%; S4 = 54.6%; and S5 = 58.2% (± 6.27%). There is no difference in the sperm kinetics and the plasma and acrosomal integrity of the membrane, after 5 hours by cooling, between the five evaluated refrigeration systems.



A004 Physiology of Reproduction in Male and Semen Technology

Assessment of scrotal termography and semen quality in buffalo bulls (*Bubalus bubalis*) raised under humid tropical environment

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Keywords: andrology, infrared thermography, water buffalo.

This study aimed assessing the surface temperatures of orbital area and scrotum of buffalo bulls using infrared thermography, monitoring the sperm quality over time and correlating surface temperatures to thermal comfort indexes. The experiment was conducted in humid tropical climate region, from April to August 2013, with maximum daily average of $31.5 \pm 0.8^\circ\text{C}$, and relative humidity of $81.3 \pm 3.8\%$. Ten water buffalo bulls (*Bubalus bubalis*, n=10) were maintained in an artificial insemination station (Cebran/UFPA, Castanhal-PA) and were evaluated each 25 days (morning: 6:00-9:00; afternoon: 12:00-15:00). Rectal temperature (RT, °C) was assessed using thermometry and surface temperatures of orbital area (ORB, °C) and scrotum (SCR, °C) were evaluated by infrared thermography. Semen was evaluated for volume, concentration, turbulence, vigor, progressive motility, sperm morphology and plasma membrane integrity, with eosin-nigrosine. Climatic data were continuously monitored and the Temperature and Humidity Index (THI) and the Index of Comfort of Benezra (ICB) were calculated. Variables with non-normal distribution were transformed to logarithmic scale. Analysis of variance was performed by the GLM SAS, version 9.3 (SAS, 2011). It was considered in the model shift (morning and afternoon) and month effects (April to August). For mean comparisons between shifts used F test and for multiple comparison of average monthly was adopted Tukey test. Correlations were calculated using Pearson test. In all analyzes was adopted $P < 0.05$. The ICB ranged from 1.96 to 2.25 and significant differences were observed for shifts and over the months ($P < 0.05$). The averages of surface temperatures were $RT = 38.2 \pm 0.5^\circ\text{C}$, $ORB = 36.1 \pm 0.8^\circ\text{C}$, $SCR = 33.3 \pm 1.1^\circ\text{C}$, which exhibited significant differences for shifts and over the months ($P < 0.05$). The gross motility and the sperm vigor were significantly different ($P < 0.05$), and a quality decrease during the warmer months and higher THI was detected. The total sperm defects ranged from $17.6 \pm 6.2\%$ and $21.2 \pm 8.2\%$, but no significant difference was observed ($P > 0.05$). The THI showed positive correlations with ORB (0.72) and ESC (0.41) ($P < 0.0001$), while the ICB was positively correlated with ESC (0.25; $P < 0.0001$). Negative and significant correlation was found between ITU and sperm plasma membrane integrity (-0.17; $P < 0.05$). Therefore, the surface temperatures of buffalo bulls and their semen quality are associated to temperature and humidity changes and suffer interference from climatic variations, justifying the management approaches to provide thermal comfort to animals in order to increase the semen quality.

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A005 Physiology of Reproduction in Male and Semen Technology

Testicular thermolysis ability of “Morada Nova” sheep under heat thermotolerance teste: preliminary results

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Keywords: andrology, hair sheep, thermoregulation.

This research aimed to evaluate thermoregulatory capacity of scrotum, of Morada Nova sheep, subjected to heat tolerance test, using infrared thermography analyses. The study was conducted from January to February 2015 in the experimental unit of Livestock Southeast, São Carlos-SP, subtropical climate region (Cwa Koppen). Seven Morada Nova males were subjected to heat tolerance test (Baccari Junior et al. 1986 Annual Meeting of the Society of Animal Science, 23, p. 316), and yours themolitics answers was evaluated during three distinct periods (T1, T2 and T3), with animal exposure to the sun and shade. In T1, animals were maintained in the shade for two hours (11:00 to 13:00), then they were exposed to direct sunlight for a period of one hour (13:00 to 14:00), featuring T2. In T3, animals returned to the shade, where they remained for an hour (14:00 to 15:00). At end of each period, rectal temperatures were measured (RT-oC) with thermometry. Testicular surfaces temperatures (°C) measured were: dorsal pole temperature (DPT), ventral pole temperature (VPT) and average testicular temperature (ATT). Gradients of temperature between the dorsal and ventral poles were calculated (GDV) and between rectal temperature and average testicular (GRT). These measurements were made with infrared thermal imager (Testo875i, Testo®, Lenzkirch, Germany). Statistical analysis consisted of evaluation of the normality of data, analysis of variance (ANOVA). The effects of time and results were expressed in mean ± SD. Significance level was 5%. Rectal temperatures and testicular surface measured on T2 (RT=39.0±0.3°C, DPT=34.0±1.2°C, VPT=33.5±1.4°C, ATT=34.0±1.1°C and GRT =4.9 ±1.0 °C) were significantly higher than observed in T1 (RT=38.3±0.3°C, DPT=33.0±1.0°C, VPT=32.1±1.0°C, ATT=32.6±0.9°C and GRT=5.7±0.9°C) and T3 (RT=38.4±0.3°C, DPT=32.5±1.8°C, VPT=31.4±2.0°C, ATT=32.1±1.7°C and GRT=6.3±1.6°C). Thus, there was no statistical difference between values observed in pre and post challenge. Only gradient between testicular poles (GDV) behaved differently from the others, with statistical difference between T2 (0.48±0.63°C) and T3 (1.1±0.79°C). These results demonstrate the thermoregulatory efficiency of Morada Nova sheep, since even after submission to heat stress situation, its surface testicular temperatures were set to baseline standard noted previously challenge. This ensures the maintenance of the physiological temperature gradient between core body and testicles, vital for normal spermatogenesis.



A006 Physiology of Reproduction in Male and Semen Technology

Cellrox Deep Red[®] is effective for detecting oxidative stress in bovine sperm: preliminary studies

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Keywords: fluorescent probe, reactive oxygen species, semen.

Reactive oxygen species (ROS) are produced physiologically by sperm cell. Cryopreservation is able to increase this production and it results in presence of antioxidants in composition of majority of extenders. The probe CellROX Deep RED[®] (Molecular Probes) was validated by our group to identify ROS in ram sperm. However, it has not been efficiently evaluated in bull sperm yet, which is the purpose of the study. For this, commercially available frozen semen from four bulls was used. Two straws of each bull (n=4) were thawed (37°C/30 seconds). The sample was centrifuged at 500 g/10 minutes to remove the supernatant (extender sperm free), stored at 5°C. The precipitate was suspended in 200 µL of Tyrode's albumin lactate pyruvate (TALP) and added to 50 µL of iron sulfate and 50 µL of ascorbic acid kept at 37°C/90 minutes maintained with open tube cap for oxidative stress induction. The sample was centrifuged at 500 g/10 minutes. The supernatant was removed and the precipitate was suspended in 300 µL of TALP. The concentration was adjusted to 20x10⁶ sperm/mL, diluting one sample in TALP (control group), and another in the stored extender. It was performed that to evaluate if the probe would be able to identify sperm with ROS in control group, since in extender group probably it would be observe a little level of oxidative stress because of antioxidants proprieties of extender. Aliquots of 200 µL of the samples (control x extender) were added with 4 µL of CellROX Deep RED[®] 1 mM and 1 µL of Hoescht 33342 0.5 mg/mL (Molecular Probes) and incubated at 37°C/30 minutes. The samples were centrifuged at 5000 g/5 minutes, the supernatant was removed and the samples were suspended in 200 µL of TALP. It was prepared a humid chamber with 4 µL of the samples and 200 cells per slide were counted. The cells were classified as absent or few presence of ROS (FEW), moderate level of ROS (MOD) and intense level of ROS (INT). FEW and INT variables were transformed and subjected to analysis of variance (ANOVA). For the variable MOD, an evaluation by nonparametric statistics was made. It was used SAS software (SAS Institute Inc., 2004) and the significance level was 5%. After the stress induction, control group showed a reduction (p=0.005) in FEW cells (5±3.84%) when compared to extender group (74.87±18.54%). There was no difference (p=0.24) between control group (68.87±4.14%) and extender group (24.50±18.26%) for MOD cells. However, control group (26.12±1.86%) showed higher (p<0.0001) INT amount of cells than the extender group (0.62±0.31%). Thus, it can be concluded that the CellROX[®] probe is able to identify ROS in bovine spermatozoa and that the extender is capable of neutralizing these species. However, more studies are being done by our group to confirm the efficiency of the ROS evaluation in bull criopreserved sperm.



A007 Physiology of Reproduction in Male and Semen Technology

Single layer centrifugation improves stallion sperm motility after storage at 15°C

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Keywords: cooling, colloid, ejaculate.

Cooling of stallion semen for transport and subsequent artificial insemination has become widespread in equine reproduction. Cooled storage prolongs the lifespan of spermatozoa, however, this condition can also induce irreversible damages to structural integrity, biochemistry and biophysics of the sperm cell (Aurich C, Anim Repro Sci, 107:268-275, 2008). In addition, there is considerable variability in sperm survival of individual stallions during the process. Single layer centrifugation (SLC) is a colloid centrifugation technique designed to select spermatozoa with good motility, membrane integrity, normal morphology and intact chromatin from the rest of the ejaculate (Morrell JM & Rodriguez-Martinez H, Open Andro J, 1:1-9, 2009), improving sperm quality. The aim of this experiment was to evaluate the effect of SLC in stallion sperm motility after cooled storage at 15°C for 8 hours. During reproductive season and after two days of sexual arrest, ejaculates were collected from ten Crioulo breed stallions (n=10) with artificial vagina and an estrus mare. After collection, the ejaculate was filtered and diluted with commercial semen extender (EquiPlus®, Minitüb GmbH, Tiefenbach – Germany) to 50x10⁶ spz/mL. Semen samples were refrigerated for 8 hours at 15°C and then submitted to two treatments: Conventional centrifugation (600G x 20') and SLC (Androcoll Equine®, Minitüb GmbH, Tiefenbach – Germany) (300G x 20'). In both protocols, the pellet was resuspended with the same extender. Progressive motility (%) was evaluated by Computer Assisted Sperm Analyzer (AndroVision® Minitüb GmbH, Tiefenbach – Germany) in three moments: 1) after collection and dilution, 2) after 8h of cooling (pre-centrifugation) and 3) after centrifugation and resuspension (post-centrifugation). Descriptive statistic and mean comparison by Kruskal-Wallis test were performed in the program Statistix9® (p<0.05). The semen diluted had shown a 68.7±4.2 mean of percentage progressive motility. Along the cooled storage period, the sperm motility mean dropped significantly (p<0.05) to 48.7±6.2 (Conventional centrifugation) and 49.9±6.0 (SLC). The sperm percentage motility after conventional centrifugation and resuspension (45.2±5.6) had no difference in comparison with the previous moment, while the SLC treatment resulted in 58.5±5.7. The mean of sperm motility after SLC was significantly higher (p<0.05) when compared with the moment pre-centrifugation. There was no statistical difference between the moment post-SLC when compared to the initial motility measured prior cooled storage demonstrating the efficiency of this method to improve the semen quality selecting the better sperm cells. Semen samples submitted to SLC had 29.4% higher progressive motility than the sample with normal centrifugation. It is suggested that these semen samples could be used for subsequently artificial insemination of mares or posterior methods to increase its shelf life. In the present experiment, it is demonstrated the beneficial effect in progressive motility of SLC after cooled storage at 15°C for a short period of time.



A008 Physiology of Reproduction in Male and Semen Technology

Comparison of blood flow on testicular artery, hormone dosage and semen quality between texel and “Santa Ines” sheep

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Keywords: hormonal dosage, sheep, spectral Doppler.

The aims of this study were to evaluate and compare testicular parameters, sperm quality and hormonal dosage between Texel and Santa Inês sheep. For this, 25 healthy animals (12 Texel and 13 Santa Ines) aged between 2 to 4 years old were used, from the Department of Animal Reproduction and Veterinary Radiology of Sao Paulo State University (UNESP – Botucatu). Testicular morphometry was initially performed (length - LEN and width - WID in centimeters) and then the spectral doppler ultrasound examination of testicular artery (pulsatility index - PI and resistance index - RI) was evaluated in the spermatic cord. Subsequently a sample of blood from each animal was collected in the morning by puncture of the external jugular vein were collected for access the testosterone dosage (DT, mg/dL) and then the semen was collected by electroejaculation. The sperm kinetic parameters were analyzed by computerized method CASA (TM% total motility, %PM - progressive motility, $\mu\text{m/s}$ VAP – average speed path, $\mu\text{m/s}$ VCL - curvilinear velocity, $\mu\text{m/s}$ VSL - progressive linear speed, %RAP - rapid sperm) and the plasma membrane integrity (MPI) was analyzed by epifluorescence microscopy. The testosterone dosage was access by kits and the analysis performed by radioimmunoassay. The data generated were evaluated by Student’s t-test and differences were considered significant at $p < 0.05$. The mean values and standard deviations found for the evaluated parameters for Texel and Santa Ines breeds are placed following, respectively: TM (81 ± 7.4 and 84 ± 17.8); PM (57.4 ± 10.1 and 62.1 ± 17.4); VAP (130.4 ± 19.6 and 131.6 ± 18.3); VCL (195.2 ± 38.1 and 181.0 ± 22.3); VSL (123.4 ± 29.4 and 111.6 ± 8.9); RAP (70.0 ± 10.2 and 74.4 ± 18.3); MPI (64.8 ± 11.4 and 61.2 ± 12.9); LEN left testicle (9 ± 1.11 and 9.5 ± 1.2); WID left testicle (6.2 ± 0.6 and 6.3 ± 0.5); LEN right testicle (9.1 ± 0.9 and 9.7 ± 1.2); WID right testicle (6.1 ± 0.7 and 6.2 ± 0.5); DT (1.08 ± 0.65 and 2.97 ± 5.6); PI left testicle (0.99 ± 0.27 and 1.16 ± 0.33); RI left testicle (0.63 ± 0.11 and 0.68 ± 0.12); PI right testicle (1.16 ± 0.46 and 1.17 ± 0.47) and RI right testicle (0.67 ± 0.17 and 0.68 ± 0.16). In conclusion there was no difference in any of the parameters evaluated between the sheep breeds.



A009 Physiology of Reproduction in Male and Semen Technology

Different *in vitro* sperm challenge and its relationship with *in vivo* bull fertility

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Keywords: Brangus, fertility, laboratory sperm challenge.

The aim of this work was to challenge the laboratory quality of thawed semen and to compare the *in vitro* results with *in vivo* semen fertility. Frozen-thawed semen of 4 different batches from the same bull, which were previously used in a TAI program were used for insemination of 332 Brangus cows. For laboratory experiment, three repetitions of each batch were performed. For each semen dose, the following procedure was accomplished: initially, the semen sample was thawed at 37°C for 30 sec (control), sperm motility was assessed by CASA and plasma membrane integrity was evaluated by propidium iodide fluorescent probe. Then, an aliquot of 150 µL of the sample was incubated in a water bath at 45°C for 40 min (thermal challenge group; GDT) and another aliquot of 150 µL of the sample was centrifuged at 500 x g (Percoll gradient 45%/90%) for 15 min (centrifugation challenge group; GDC). The centrifuged semen was also subjected to another thermal challenge, being incubated (water bath) at 45°C for 40 min (centrifugation + thermal challenge group; GCDT). At the end of each challenge (GDT, GDC and GCDT), the same laboratory tests used for control group were repeated. The field data were analyzed by GLIMMIX of SAS and laboratory data by analysis of variance in GraphPad INSTAT. Significance level of 5% was established. No difference ($P>0.05$) between AI technician, BCS or batches (B) was observed for conception rate (CR). The following CR were observed for each batch: B1 = 48.9% (44/90); B2 = 44.2% (23/52); B3 = 55.5% (40/72); B4 = 43.2% (51/118). Although no statistical difference was observed between batches, numerically higher CR was observed for B3 compared to B4. According to CASA results, it was interesting to note that B4 was the batch that presented lower ($P<0.05$) percentages of Progressive Motility (PM) both after thawing (control: 47.2 ± 8.5) and after all sperm challenges (GDT: 40.0 ± 4.6 ; GDC: 45.7 ± 7.3 ; GCDT: 4.7 ± 7.2) compared to B3 (control: 63.0 ± 5.3 ; GDT: 56.0 ± 1.7 ; GDC: 64.2 ± 12.5 ; GCDT: 7.7 ± 3.8). In addition, while B3 and B4 demonstrated similar percentage of plasma membrane integrity (MPI) in control (T3 = 66.7 ± 1.3 and T4 = 65.2 ± 3.3), the semen of B3 demonstrated higher ($P<0.05$) percentage of MPI (37.2 ± 2.5) than B4 (26.7 ± 3.3) after passing through the greatest challenge of this *in vitro* experiment (GCDT). According to the results, it was concluded that the semen of batch 3 was the most resistant to the proposed laboratory challenges, especially when compared to batch 4. Therefore, the present study suggests that to submit seminal samples to a laboratory challenge before to perform an *in vivo* semen quality assessment seems to be an interesting alternative for define semen batches that may present greater reproductive performance of field fertility.

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A010 Physiology of Reproduction in Male and Semen Technology

Viability and longevity of bovine epididymal spermatozoa after cryopreservation

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Keywords: bovine, kinetics, semen.

The recovery of epididymal spermatozoa (EP) and its use in assisted reproductive techniques have an important role in multiplication of genetic material from bulls that died suddenly and/or have acquired reproductive failure. However, to maximize the *in vivo* and *in vitro* use, a better knowledge about the physiologic behavior of those spermatozoa is necessary. The aim of this study was to characterize the viability and longevity of cryopreserved sperm recovery of epididymal tail, for 24 h. Epididymis (EP) and ejaculated (EJ) sperm were recovered from the same bulls of Gir breed (n=7) and were cryopreserved. Three groups were used: EJ, EP and EP that were exposed for 10 min at 39°C in a pool of seminal plasma (EPP). The three sperm groups were selected in Percoll gradient 45:90% and were incubated at 39°C for 24 h in SP-TALP buffer medium in a final concentration of 2×10^6 spermatozoa/ml. Sperm samples from the three groups (EJ, EP and EPP) were removed at 0, 3, 6 and 24 h of incubation and were evaluated for total (TM) and progressive motility (PM) (CASA), morphology (phase contrast), capacitation (chlortetracycline-CTC), plasma membrane integrity, assessed with propidium iodide (PI) and 6-carboxyfluorescein diacetate (C-FDA) and acrosomal integrity technique Peanut agglutinin (PNA conjugated to FITC). Data were analyzed by GLIMMIX procedure using SAS program ($P \leq 0.05$). At 6 h of incubation EJ group showed a bigger decrease on MT (33.9 ± 8.8), MP (EJ 23.3 ± 8.1), IMP (16.4 ± 3.4), IA (19.9 ± 3.2), and percentage of non capacitated sperm (70.3 ± 3.7) in relation to EP and EPP groups. The EP and EPP groups, at 6 h of incubation showed MT (56.7 ± 9.3 and 40.6 ± 9.2), MP (47 ± 9.6 and 35 ± 9.2), IMP (29 ± 4.3 and 28.2 ± 4.2), IA (31.6 ± 3.8 and 31.6 ± 3.8), and percentage of non capacitate sperm (80.9 ± 3.5 and 81.0 ± 3.5) similar to those observed at 0h. At 24 h of incubation, all groups presented similar MT (EJ= 0.5 ± 1.4 , EP= 9.8 ± 5.7 ; EPP= 10.6 ± 5.9), MP (EJ= 0.4 ± 1.3 ; EP= 6.5 ± 4.9 and EPP= 8.2 ± 5.5), IMP (EJ= 6.7 ± 2.3 ; EP= 10.3 ± 2.8 and EPP 10.7 ± 2.8) and IA (EJ= 7.1 ± 2.0 ; EP= 10.3 ± 2.4 ; EPP= 11.1 ± 2.5). However, the percentages of non capacitate sperm was lower on the EJ group (68.0 ± 4.6) than on the other groups, EP (76.0 ± 3.8) and EPP (80.5 ± 3.5). It can be concluded that after thawing, epididymis sperm were able to maintain their quality for a longer period than the ejaculated, suggesting they have higher cryo resistance than the ejaculated sperm. In addition, exposition of EP to seminal plasma did not affected EP viability and/or longevity.

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A011 Physiology of Reproduction in Male and Semen Technology

Effects of cryopreservation on motility of Nordestino breed stallion sperm

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Keywords: CASA, equine, kinematic.

Studies show that the racial factor directly affects the sperm cell resistance to cryopreservation. In addition, in some stallions, the low quality of frozen semen appears to be related to the presence of glycerol in freezing media (Alvarenga; Papa 2011, Spermova 1:7-10). Although Nordestino breed is essential in the Caatinga, there are few studies on cryopreservation of semen of stallions of this breed. This study aimed to analyze the motility of the sperm of stallions of the Northeastern race after cryopreservation. This study was approved by the CEDEP of UNIVASF (Protocol nr. 0006/161012). Nineteen ejaculates used two stallions (N = 10 and N = 9 ejaculates, respectively) were collected by artificial vagina and female in oestrus. The ejaculates showed milky white-gray coloring, sui generis odor, and the total sperm motility (TM%) of 80.91 ± 9.29 and progressive (PM%) of 59.24 ± 11.45 . After collection, the samples were diluted (1:1) with Botu-Semen® (Botupharma Botucatu, SP), centrifuged in 50 ml tubes at 2200 rpm for 15 min and the pellet resuspended with Botu-crio® (Botupharma, Botucatu, SP) to determine sperm concentration using the photometer Spermacue® (Minitube, Berlin, Germany). After this, the semen was packaged in 0.5 ml straws and kept at 5°C for 90 min and then packaged into 0.5 ml straws, frozen in liquid nitrogen vapor for 20 min before being plunged into liquid nitrogen for storage. Semen was thawed in a water bath at 37°C for 30 s. The TM and PM were determined using the CASA® (Minitube, Germany, Berlin). Aliquots (8 µL) of thawed semen were removed, placed between slide and cover slip, pre-heated to 37°C, and evaluated for sperm motility. Data were analyzed using ANOVA and SNK test ($P < 0.05$; SAEG, UFV 1997). The TM and PM of sperm after thawed was 34.08 ± 12.57 and 20.53 ± 12.82 , respectively. Preliminary results indicate that the Stallions Nordestino breed analyzed have good semen freezability and keep the parameters of total and progressive sperm motility.

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A012 Physiology of Reproduction in Male and Semen Technology

The effect of heat shock on bovine sperm motility and cytoskeleton organization

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Keywords: heat shock, immunofluorescence, sperm.

Bull heat stress increases scrotal and testicular temperature, reduces sperm quality, motility and fertilization ability. Sperm changes in motility are directly associated with organization of cytoskeletal proteins. Therefore, the objective of this study was to determine the effect of heat shock on bovine sperm motility and cytoskeleton organization. A pool of three *Bos indicus* semen straws was used for each replicate (N = 6 replicates). Semen straws were purified on Percoll gradient. Sperm sample (1×10^6 spermatozoa/ml) was subjected to motility evaluation and immunofluorescence for localization of microtubules and the molecular motor dynein immediately after Percoll gradient (0 h Control) and after SP-TALP incubation at Control (38.5°C) and Heat Shock (41°C) for 4 hours. Sperm motility was assessed every hour during the incubation period. Samples of the 0 hour control and after 4 hours incubation were processed with anti- α -tubulin mouse monoclonal IgG, anti-cytoplasmic dynein rabbit polyclonal IgG, Alexa Fluor 488® goat mouse IgG, Alexa Fluor 555® goat mouse IgG and Hoechst 33342 for sperm tubulin, dynein and DNA localization. Tubulin (N= 600 sperm/treatment) and dynein (N = 300 sperm/treatment) morphological localization and pixel fluorescence intensity was determined with Image J software version 1,49j. Non-parametric data were analyzed using the Wilcoxon test of the statistical package SAS. Zero hour control (not incubated) sperm motility was higher than all the other groups ($73.12 \pm 3.6\%$, $P < 0.05$). Sperm incubation at 38.5°C affected ($P < 0.05$) sperm motility at different incubation times ($68.12 \pm 3.6\%$, $53.75 \pm 3.6\%$, $41.25 \pm 3.6\%$ and $27.12 \pm 3.6\%$ for 1, 2, 3 and 4 hours, respectively). Similarly, incubation of spermatozoa at 41°C ($P < 0.05$) reduced sperm motility over time ($52.5 \pm 3.6\%$, $43.75 \pm 3.6\%$, $20.62 \pm 3.6\%$ and $3.62 \pm 3.6\%$ for 1, 2, 3 and 4 hours, respectively). Heat shock of 41°C reduced ($P < 0.05$) sperm motility as compared to control 38.5°C at all incubation times. Immunofluorescence indicated that tubulin was localized at the sperm tail insertion and along the tail. Tubulin pixel fluorescence intensity increased ($P < 0.01$) with incubation and temperature (13.6 ± 0.29 , 15.2 ± 0.28 , 15.4 ± 0.28 arbitrary units (AU) for 0 hour control, 38.5°C and 41°C, respectively). The molecular motor dynein was localized along all the sperm cell. Similarly, dynein pixel fluorescence intensity increased ($P < 0.0001$) with incubation (4.2 ± 0.5 , 26.8 ± 0.5 , 26.2 ± 0.5 AU for 0 hour, 38.5°C and 41°C, respectively). In conclusion, incubation and elevated temperature reduced sperm motility and affected the pattern of cytoskeletal proteins organization.



A013 Physiology of Reproduction in Male and Semen Technology

Effects of plasma testosterone and total protein of seminal plasma on the sperm parameters of semen donors Nelore bulls

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Keywords: seminal plasma, testosterone, total protein.

Researches aiming to relate testosterone concentration (T) and protein fractions of seminal plasma (SP) are common, mainly those related to sperm production and freezability, respectively. However, few studies have sought to establish an association between T or SP total protein (TP) and spermatoc variables usually used to evaluate sperm quality in Semen Collecting and Processing Center. Thus the goal of this study was to verify the influence of plasmatic T and TP of SP on the sperm parameters from semen donors bulls. Forty ejaculates from 8 Nelore bulls were collected by artificial vagina. The sperm samples were examined for motility (M, %), vigour (V, 0-5), concentration (C; spermatozoa/mL) and sperm morphology (%; major defects [MD], minor defects, [mD] and normal [N], before freezing. The SP was obtained by semen centrifugation at 700 x g/10 min. The blood (five samples per animal) was collected immediately after semen collection through the jugular vein puncture. Samples of T and TP (kept at -86o C until using) were measured using commercial kits (DPC- Diagnostic Products Co®, EUA; Pierce, EUA, respectively), according to manufacturer's instructions. The results of T and TP were expressed in values of mean±standard deviation. The correlation coefficient of Spearman was used to verify possible correlations between spermatoc variables and T or TP, with P<0.05 taken as significant. Data of T and TP were 684.46±212.01 and 37.30±1.7, respectively. Significant correlations just were observed between TP and spermatoc variables, such as: vigour (r = 0.3818; p<0.015), MD (-0.5004; p<0.001), mD (r = -0.3240; p<0.041) e N (r = 0.443; p<0.005). On the other hand, there was tendency of correlation between TP and M (r = 0.266; p<0.08). However, no significant correlations were found between the spermatoc variables and T. In conclusion, under these experimental conditions, the concentration of TP of SP was effective in determining best results of sperm quality of important variables (V, MD, mD, and N), suggesting that TP acts as a modulator agent for these variables, through a mechanism not yet established. Nerveless, other investigations with the largest number of animals need to be carried out to confirm these findings, which might lead to the determination of useful marker to monitor sperm quality of semen donors Nelore bulls.



A014 Physiology of Reproduction in Male and Semen Technology

Efectiveness of distilled water for hipoosmotic swelling test in cryopreserved ovine semen

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Keywords: frozen sêmen, hipoosmotic swelling test, ovine.

The hipoosmotic swelling test (HOST) is interesting for the evaluation of functional integrity of sperm plasma membrane, also being an affordable and easy method, especially when using distilled water as hypoosmotic environment. The HOST has been used as evaluation protocol of sperm viability of many species; however, the use of distiller water as hipoosmotic solution was not standardized for ovines. Besides, the effectiveness of distilled water with traditional hipoosmotic solutions, such as citrate-fructose, has not been compared. The aim of this study was to analyze the reactivity of post-thaw sperm cells of Santa Ines and Dorper rams in distilled water and citrate-fructose (100 mOsmol/L) hipoosmotic solution, both at different dilution rates. Thirty semen samples (20y Santa Ines and 10 Dorper rams) were used. After the thawing, the kinetic sperm parameters were analyzed (total motility-TM, progressive motility-PM and sperm vigor-VIG). Aliquots were collected for the supravital test with dye eosin (EOS); the sperm morphology was analyzed and the percentage of bent tails (BT) calculated. The HOST followed the dilution: one part of semen to 10 (HOST G1), 20 (HOST G2) and 50 (HOST G3) parts of solution. The same proportions were maintained for distilled water as following: one part of semen to 10 (HOST G4), 20 (HOST G5) and 50 (HOST G6) parts of solution. The percentage of HOST-reactive spermatozoa was determined by subtracting the percentage of spermatozoa with HOST-induced bent tails (BT) from the BT obtained right after thawing. These evaluations were carried out in phase contrast microscopy (1000x) and one hundred cells were analyzed per semen sample. All the statistical analysis were performed by using the SAS software, version 5.0 (1996) (MEANS and GLM Procedure – SNK test, with $P < 0.05$). The post-thawing values were for total motility: 63.3%; progressive motility: 58.3%; vigor: 3.1; bent tails: 18.4%; sperm viability by the supravital: 42.9% and HOST: 32.4%. The averages observed in the hipoosmotic test were: HOST G1 (36.1%); HOST G2 (34.5%); HOST G3 (34.6%); HOST G4 (27.2%); HOST G5 (30.0%) and HOST G6 (32.2). Despite the numerical variations among the HOST groups, the rates did not differ ($P > 0.05$) significantly. The findings demonstrate that the HOST with distilled water is effective and can be used for the evaluation of post-thawing ovine sperm viability.



A015 Physiology of Reproduction in Male and Semen Technology

Estimating the fertilizing ability of collared peccaries (*Pecari tajacu*) sperm by analyzing its interactions with swine oocytes

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Keywords: semen, *Tayassu tajacu*, wild pig.

Conventional tests that evaluate sperm quality do not have the ability to measure the fertilizing potential of a sample. The aims of this study were to evaluate the binding capacity of collared peccaries (*Pecari tajacu*) sperm using the heterologous *in vitro* interaction test with swine oocytes from antral follicles, and establish the relations between sperm parameters and the binding test. Thus, a total of 11 ejaculates from adult individuals collected by electroejaculation was evaluated for motility, vigor, viability, normal morphology, kinetic motility parameters by computerized assisted semen analysis (CASA), membrane functionality and integrity. Moreover, 11 samples were analyzed by the *in vitro* interaction test using swine oocytes at 38.5°C and 5% CO₂ for 18 h. After this period, the oocytes were washed and labeled with Hoechst 33258 (10 µg/mL) and visualized by fluorescence microscopy. The estimated fertilizing capacity was analyzed according to the number of bound sperm and/or penetrated the zona pellucida. All the data were expressed as mean ± SD and a simple linear regression model was used to identify associations between sperm-oocyte interactions (dependent variables) and sperm parameters (independent variables). Thus, the mean values for semen parameters evaluated by conventional analysis and CASA were as expected for the species. In the *in vitro* interaction test, we verified that all the swine oocytes (100%) presented bound sperm to zona pellucida, but only 19.85 ± 5.5% oocytes presented penetrated sperm. Additionally, an average of 39.4 ± 4.6 bound sperm/oocyte and 2.5 ± 0.7 penetrated sperm/oocyte were found. Probably, the composition of the zona pellucida of swine oocytes is similar to the peccaries, thus suggesting its use as heterologous substrate for the evaluations of sperm penetration capability for peccary. Among the sperm parameters, only the straightness rating – STR presented association to the number of bound sperm (R = 61.7%; P < 0.05). Such parameter is related to progressive sperm, indicating the hyperactivation, and it is related to the fertility. In conclusion, the *in vitro* interaction test at using swine oocytes do not present marked relations to sperm parameters currently evaluated in collared peccaries. Further studies are needed to enable the use of heterologous substrates as accurate indicator of fertility for the species.

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A016 Physiology of Reproduction in Male and Semen Technology

Seminal quality comparative study between pets and working animals of the Australian Cattle Dog breed

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Keywords: Australian Cattle Dog, dog, semen.

The objective of this study was to evaluate seminal characteristics in dogs of the Australian Cattle Dog breed. The animals were divided into two groups: Group I (GI) pets who do not perform regular physical activity (N1 = 4) and Group II (GII) animals used daily in animal husbandry work (grazing) on farms (N2 = 3). We evaluated data scrotal circumference and seminal characteristics of seven dogs, evaluating three ejaculates from each animal (N = 21 ejaculates). The collection of the semen was performed by digital manipulation; being only used the second fraction of the ejaculate for evaluation. The ejaculates were evaluated for macroscopic characteristics: volume (VOL) and microscopic: progressive motility (MOT), vigor (VIG), concentration (CONC) and morphology (MORF). For testis size were carried out scrotal circumference measurements (CE), right testicle long (CTD) and left (CTE) and wide right testicle (LTD) and left (LTE), with the help of a tape measure and calipers, respectively. The means and standard deviations for the variables weight and age of the animals were 22.18 ± 3.43 kg and 4.57 ± 2.63 years, respectively. For the evaluation of variables related to semen characteristics we used the statistical model that looked at the group of fixed effects (GI vs. GII), and random effects of animal and residual. Analyses were performed considering structure of repeated measures in the same animals, by using the PROC MIXED SAS (SAS INC, 2004). For CONC variable, data were submitted to logarithmic transformation. For the EC variables, CTD, CTE, LTD and LTE the average estimates, and the standard deviations found were respectively 6.31 ± 0.95 cm, 1.75 ± 0.27 , 1.85 ± 0.25 , 2.30 ± 0.36 and 2.64 ± 0.57 cm. For the variables related to seminal quality, average estimates were observed and standard deviation: VOL, 1.11 ± 0.21 and 2.01 ± 0.25 mL; MOT, 82.08 ± 4.32 and $73.33 \pm 4.99\%$; VIG, 4.00 ± 0.15 and 3.22 ± 0.19 ; Pathol, 7.08 ± 0.91 and $6.1 \pm 1.05\%$, and CONC, 8.43 ± 0.09 and 8.36 ± 0.11 sptz/ejaculate. The analysis of variance revealed a significant effect ($P < 0.05$) for the VOL (GI: 1.12 ± 0.22^b ; GII: 2.01 ± 0.25^a mL; $P = 0.0441$) and VIG (GI: 4.0 ± 0.17^a ; GII: 3.22 ± 0.19^b ; $P = 0.03$) variables. The results of this study suggest that although the two groups present semen quality, companion animals (GI) exhibited higher values for vigor compared to animals used for work (GII). As for the VOL variable, the working animals (GII) presented higher values than pets (GI).



A017 Physiology of Reproduction in Male and Semen Technology

Significance of morphofunctional semen evaluation of buffalo bulls used for timed artificial insemination

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Keywords: buffaloes, cryopreservation, functional tests.

Around 50% of sperm are lost during bovine semen cryopreservation process (Arruda, R. P. *Biotecnologia da reprodução em bovinos*. 1º Simpósio Internacional de Reprodução Animal Aplicada, p.166-179). In such context, the development of laboratory tests aiming to predict more accurately the performance of cryopreserved semen has been target of field researches in AI programs (Arruda, R. L. *Ver. Bras. Reprod. Anim.*, v.34, p.168-184, 2010). The present work aimed to demonstrate the influence of morphological and functional semen analysis for the success of fixed time artificial insemination programs (FTAI) in buffaloes using cryopreserved semen. One hundred and five buffaloes [59.1±2.4 days post-partum and average body score condition of 3.7±0.0 (1-5)] were synchronized to TAI at a random day of the estrous cycle (D0; 16:00). All the buffaloes were treated with a P4 intravaginal releasing device (1g progesterone; Sincrogest®, Ourofino) and 2.0 mg of estradiol benzoate im (Sincrodiol®, Ourofino). On D9 (16:00), females received 0.53 mg im of PGF2α (Cloprostenol, Sincrocio®, Ourofino) and 400 IU eCG im (Novormon®, MSD Animal Health), followed by the removal of progesterone device. On D11 (16:00), 10 µg of Buserelin Acetate (GnRH, Sincroforte®, Ourofino) were administered im. The TAI was performed 16 hours after the application of GnRH (D12; 8:00). Sixty four (64) semen straws of bull 1 and 41 straws of bull 2, from the same batches, were used in the same TAI protocol. Bulls were selected according to the following criteria: motility>50%, vigor>3, concentration higher than 10 million sperm/straw, total defects<30%. Although approved by such criteria, there was a difference of 8.5% between pregnancy rates of both bulls (bull 1: 71.9; bull 2: 63.4). Three semen samples of each batch were thawed and subjected to functional semen analysis (ie, plasma membrane integrity - Eosin-Nigrosine; acrosome integrity - Fast-Green/Rose Bengal; mitochondrial activity - Diaminobenzidine; DNA fragmentation - SCSA and lipid peroxidation - TBARS). Despite the lower percentage of motile cells (bull 1: 56.7±3.3% vs. bull 2: 65.0±2.9%), the higher fertility bull showed similar number of mobile sperm per straw (18.5% vs. 18.4%). However, the higher fertility bull showed a lower percentage of major defects when compared to lower fertility bull (7.0±0.6% and 20.3±0.9, respectively). There were also a higher percentage of cells with intact plasma acrosome membranes in the highest fertility bull (bull1: 80.7±4.1% and 95.3±0.3%; bull 2: 63.7±2.4% and 76.7±0.9%, respectively). No differences were found on mitochondrial activity, DNA fragmentation or lipid peroxidation. Preliminary results indicate that together physical analysis, sperm morphological and functional tests may be essential for the assessment of post-thaw fertility in buffalo TAI programs.



A018 Physiology of Reproduction in Male and Semen Technology

The influence of different methods of frozen-thawed ovine spermatozoa selection on sperm capacitation and viability after incubation

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Keywords: ram, sperm longevity, sperm selection.

Sperm capacitation is an essential event for fertilization; however, it decreases the sperm lifespan and viability. The aim of this study was to evaluate the effects of four sperm selection techniques on sperm capacitation and viability after incubation. A pool of frozen-thawed sperm from 10 Santa Inês rams was used. The samples were submitted to one of the following sperm selection techniques: sperm washing, Percoll gradient, mini-Percoll gradient, Swim-up and control group. At mini-Percoll technique, was used 400 microliters of 90% and 45% gradients and a centrifugation at 500 xg for 5 minutes. In Percoll, was used 1 mL of each gradient and a centrifugation at 700 xg for 10 minutos. During Swim-up, the sperm was incubated in 1 ml of SPERM-TALP for 45 minutos in humidified atmosphere at 37.5°C. Finally, at sperm washing the sample suffered centrifugation at 300 xg for 8 minutes, using SPERM-TALP. At the end of each treatment, the selected spermatozoa were incubated at 37°C for 1 h, 2 h, and 3 h. Viability was assessed using acridine orange-propidium iodide combination by computer-assisted sperm analysis. Capacitation status was evaluated using chlortetracycline staining and observed under epifluorescence microscopy. Data were analyzed by ANOVA, followed by Tukey test ($P<0.05$). After 3 h of incubation, the capacitated sperm was decreased ($P<0.05$) in all treatments. The capacitated sperm rate was similar ($P>0.05$) among Percoll (36%), mini-Percoll (34%) and Swim-up (30%), and were lower ($P<0.05$) than control group (47%) and sperm washing (41%), regardless of the time of incubation. The non-capacitated sperm percentage was higher ($P<0.05$) at 0 h (12%) and decreased after 3 h (1.5%), in all treatments. Regarding to acrosome reacted cells, there was an interaction ($P<0.05$) between incubation and sperm selection treatment. The acrosome reacted spermatozoa showed a lower percentage ($P<0.05$) at 0 h (50%) and 1 h (53%) and higher after 3 h (64%). Percoll and mini-Percoll were higher about acrosome reacted spermatozoa ($P<0.05$; 60% vs. 61%), whereas control group was the lowest (49%). There was an interaction ($P<0.05$) between incubation and treatment in sperm viability. Viability assays revealed that 0 h resulted in a higher rate (17.5%; $P<0.05$) of membrane integrity, after all treatments. Swim-up treatment showed a higher membrane integrity rate (17.4%; $P<0.05$), regardless of time of incubation. In conclusion, the incubation affects the capacitation status and viability of frozen-thawed ovine sperm. Sperm selection increases the acrosome reacted cells rate and Swim-up allows better viability during incubation.

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A019 Physiology of Reproduction in Male and Semen Technology

***In vitro* production of bovine embryos using frozen semen with or without the presence of seminal plasma**

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Keywords: centrifugation, filtration, Nellore.

Seminal plasma, that mixes with sperm in the ejaculate, and serves as a means of transportation to the female genital tract, has been described as beneficial and harmful to the spermatozoa. There are reports of negative influence of seminal plasma on the storage of semen, due to components harmful to sperm viability. An alternative to reduce the concentrations of seminal plasma of the ejaculate is its semen centrifugation or filtration. However, several studies have reported apparent injury to bovine sperm damaging fertilization by the method of centrifugation. The objective of this study was to evaluate rate of bovine embryos produced *in vitro* (IVP) using frozen semen with or without the presence of seminal plasma. Semen used for IVP was obtained from 31 Nelore bulls collected by electroejaculation. The semen sample was divided into three equal aliquots and the treatments performed as Campanholi et al. (VII Congresso Interinstitucional de Iniciação Científica (CIIC), Campinas, São Paulo, Brasil, 2013, p.1-8). Treatment 1 (Conventional) constitutes the dilution of semen in the traditional freezing to the final concentration of 60×10^6 spz/mL with extender BotuBov® (BotuPharma®, Botucatu, Brasil). Treatment T2 (Centrifuged) involved the centrifuging for 10 minutes at 600xg (2200 rpm) for removal of seminal plasma. Treatment T3 (Filter) was performed by the Sperm Filter® (filtration device; BotuPharma®, Botucatu, Brasil). After centrifugation and filtration of semen, spermatozoa were resuspended with extender BotuBov® at the same concentration of T1. After treatments semen was packaged at room temperature in 0.5 mL straws and frozen using the machine TK 4000® (Tetakon®, Uberaba, Brasil). Bovine oocytes to IVP were obtained from follicular aspiration from slaughterhouse ovaries. The statistical analyzes were performed in SAS PROC GLM, using 5% significance. No differences among treatments were detected in cleavage rate, T1=82.1 ± 0.83% (3748/4570), T2=82.05 ± 0.83% (3719/4535) and T3=84.01 ± 0.83% (3786/4507). The rate of embryos evaluated on D7 was higher (P<0.001) in T1 (31.30 ± 1.07%, 1430/4570) and T3 (32.3 ± 1.07%, 1476/4570) when compared to the treatment T2 (26.55 ± 1.07%, 1204/4535). The T3 (24.0 ± 1.05%; 1082/4570) treatment had higher (P<0.001) hatched blastocyst rate than T2 (18.03 ± 1.05%; 854/4535) and rate similar to T1 (21.22 ± 1.05%; 970/4570). Thereat, seminal plasma removal using SpermFilter® not changed the rate of embryos on days D7 and D9 when compared with conventional frozen semen.

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A020 Physiology of Reproduction in Male and Semen Technology

Deep cervical insemination by cervical catheterization in dairy ewes

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Keywords: ovine, cervical catheterization, clamping

The transcervical artificial insemination (AI) technique is limited in ewes because the cervical rings morphology prevents or impairs the introduction of the AI pipette. The positive correlation between the cervical penetration ease at AI and pregnancy rate justifies the development of techniques that allows a deeper deposition of semen into the cervix. This study aimed to compare the pregnancy rates after superficial versus deep cervical AI. The deep AI was performed due by clamping and traction of the vaginal fornix, and the catheterization of the cervix using the semen applicator (Alta genética®). Fifty ewes were synchronized using vaginal progesterone pessaries and injection of 350 IU eCG, combined with 0.125 mg of prostaglandin at pessary removal (day 12). Estrus detection started 12 h after pessary removal, and AI was performed 12 h after estrus detection, being the ewes randomly allocated into each of the 2 experimental groups. Twenty-seven ewes were inseminated using the superficial cervical AI technique, and 23 were bred by the deep cervical technique (only considered if the catheter would pass through the third cervical ring). For both techniques, the female hindquarters were raised at a 45° angle in relation to the ground. With the aid of a vaginal speculum the cervix was located, and the semen was deposited either in its entrance (superficial cervical AI) or after the third cervical ring, in this case using topical anesthesia to clump the fornix and traction the cervix (deep cervical AI). The semen used was collected from 4 rams (Lacaune and Milchshaff breed), diluted 1 + 3 in Tris egg yolk, loaded in 0.25 mL straws and cooled at 5 °C. Starting from room temperature, the semen was cooled at a 0.5°C / minute rate. The pregnancy diagnosis was performed 30 days after AI. Data were compared by the chi-square test (Excel, Microsoft), with 5% of significance level. A great variation in the progression of the cervical catheter was observed after clamping the fornix. From 23 ewes submitted to deep IA, it was possible to overpass the entire cervix in 9, whereas in the remainder 14, the deep insemination (after the third cervical ring) was always enabled, without semen reflux. The pregnancy rate was 33.3% in superficial cervical AI, and 52.2% in deep cervical AI. Data do not differ with at 5% of significance level, but it was observed a difference at 9% level suggesting a tendency of better performance with deep insemination. News trials are going to be run in order to increase the number of animals and enable more robust conclusions.



A021 Physiology of Reproduction in Male and Semen Technology

***In vitro* heat stress model for *Bos taurus taurus* sperm cells**

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Keywords: *in vitro* heat stress, SP-TALP, TL-semen,

Bull heat stress increased testicular temperature reducing sperm production, motility and increasing the percentage of abnormal sperm morphology. Establishment of an *in vitro* heat stress model for bull sperm requires medium validation to evaluate cellular function based on sperm motility and mitochondrial activity. SP-TALP (Tyrode-albumin-lactate-piruvate) and TL-semen chemical composition are similar, except for BSA (Bovine Serum Albumin), lactate and piruvate present on SP-TALP (Bavister, Biol Reprod, 16:228-237,1977). Therefore, the objective of this study was to establish an *in vitro* heat stress model for *Bos taurus taurus* sperm evaluating motility and mitochondrial activity. Frozen semen straws (n=30) from four Holstein bulls were used (N= pool of 3 straws/replicate). Samples were evaluated immediately after Percoll Gradient (0 hour) and after SP-TALP and TL-semen incubation at 35°C, 38.5°C and 41°C during 4 hours. Sperm motility was determined at 0, 1, 2, 3 and 4 hour incubation and mitochondrial activity (Hoechst 33342/MitotrackerRed) at 0 and 4 hour incubation. Data were submitted to ANOVA and non parametric data were analyzed by Wilcoxon using SAS 9.0. SP-TALP sperm incubation at 35°C (54 ± 4.07%); 38.5°C (53 ± 4.07%) and 41°C (47 ± 4.07%) for 2 hours did not affect motility as compared to 0 h (64 ± 4.07%). However, SP-TALP sperm incubation at 41°C for 3 hours reduced (34 ± 4.07; P < 0.02) sperm motility as compared to 0 h (64 ± 4.07%). TL-semen sperm incubation at 41°C for 1 h reduced motility (22 ± 3.87%; P < 0.05) as compared to 35°C (43 ± 3.87%; P < 0.05) and control 0 h (46 ± 3.87%). Incubation of sperm at 38.5°C (1.42 ± 0.017 arbitrary units (AU), P = 0.05) and at 41°C (1.41 ± 0,017 UA; P < 0.05) for 4 hours reduced mitochondrial activity as compared to 35°C (1.48 ± 0.017 AU; P < 0.05) regardless of medium. Mitochondrial activity of sperm incubated in SP-TALP medium (1.44 ± 0.014 AU) was superior (P < 0.05) than TL-semen (1.43 ± 0.014 AU) regardless of temperature. These observations suggested that important medium compounds for sperm function are lost in sperm incubated in TL-semen, but it was maintained if incorporated in SP-TALP. In conclusion, *in vitro* heat stress model using SP-TALP medium provided enriched energy substrate for sperm motility and mitochondrial activity.



A022 Physiology of Reproduction in Male and Semen Technology

The use of cushion solution during sperm *in vitro* selection reduces oxidative stress

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Keywords: cushion fluid, IVP, spermatozoa.

Despite advancements and modifications on the Percoll discontinuous density gradient method for sperm selection *in vitro*, the centrifugation step in this technique is liable to cause damage to sperm cells. This process may induce the production of reactive oxygen species (ROS) and decrease antioxidant defenses. With the view to minimize the potential damage caused by the centrifugation, a cushioned technique was developed, using a colloid (cushion media) for processing the equine and porcine pre-freezing semen. However, studies using this technique for selecting bovine sperm for IVP are nonexistent. This study aimed to assess the quality of sperm through the process of sperm selection by mini-Percoll gradient modified method (Guimarães et al., *Anim Reprod Sci*, v.146, p.103-10, 2014), using the cushioned during centrifugation, by analyzing the production of reactive oxygen species and total antioxidant capacity. Six replicates were performed, where straws of a *Bos taurus* bull were thawed and divided into four treatment groups: Control (C) with only the Percoll discontinuous density gradient; treatment C1, which was added 150µL of cushioned media (CushionFluid® - Minitube, Tiefenbach, Germany) under the gradient in the first centrifugation; C2, with the addition of the same amount of the colloid in the second centrifugation and C1-2 with both cushioned centrifugation. After the selection process, the semen samples were designed for biochemical assays. The ROS levels were determined by a spectrofluorimetric method using 2',7'-dichlorofluorescein diacetate (DCF-DA), with the results expressed in units of fluorescence (UF) (Loetchutinat et al., *Radiat Phys Chem*, vol. 72, p. 323-31, 2005); the total antioxidant capacity was determined by reducing ferric antioxidant potential (FRAP), using a standard curve of a compound with a known antioxidant activity, and the results are expressed in µg equivalent of ascorbic acid (Benzie and Strain, *Anal Biochem*, v. 239, p. 70-6, 1996). The data were evaluated by ANOVA and compared with Duncan test ($P < 0.05$). The control group showed increased production of ROS when compared to treatment with Cushion Fluid® (C1, C2 and C1-2) (0.352; 0.270; 0.267 and 0.258 UF, respectively). The antioxidant capacity of the C2 group (44.27) was lower than the treatments C and C1 (58.6 and 58.84) and similar to C1-2 (50.4). These results suggest that sperm selection by Percoll discontinuous density gradient with Cushion Fluid® decreases the ROS levels whereas the sperm total antioxidant capacity was reduced in the group that cushioned method was used in the second centrifugation.



A023 Physiology of Reproduction in Male and Semen Technology

Use of melatonin and ferulic acid as promoters of cryopreserved equine sperm

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Keywords: antioxidants, oxidative stress, stallion.

Reactive oxygen species can be responsible for causing damage to the membranes of sperm, DNA fragmentation, among other factors influencing fertility especially in cryopreservation. Melatonin (MEL) is a potent antioxidant amphiphilic (hydro and fat soluble), which makes it able to penetrate any cell compartment. The ferulic acid (FA) is a phenolic compound that exhibits a wide range of therapeutic effects against various diseases due to their potent antioxidant effect. This study aimed to evaluate the effect of antioxidants FA and MEL in cryopreservation of equine semen. Five ejaculates from four stallions were used. Among the treatments, we used two concentrations of each antioxidant (MEL 2 mM, MEL 1 μ M, FA 0.5mM and FA 1.2mM) beyond the control (conventional freezing extender BotuCrio[®] - Botupharma, Botucatu, Brazil), totaling five treatments. The parameters analyzed were sperm kinetics with the CASA system (SCA program - Sperm Class Analyzer[®]), sperm morphology by DIC, plasma and acrosomal membrane integrity mitochondrial membrane potential, using fluorescent probes PI, Hoechst 33342, FITC-PSA and JC- 1 and production of reactive oxygen species (ROS) by the sperm with the fluorescent probe CellRox Deep Red[®]. Comparisons between treatments were performed by generalized linear model (PROC GLM) of SAS (version 9.3) and the differences between them were located with the Duncan test. The probability of $P \leq 0.05$ was considered significant. The results for the motility characteristics were significant differences in some aspects, but no treatment was superior to the control. The evaluation of sperm morphology showed a decrease in major defects in the samples treated with MEL 2 mM, MEL 1 μ M and FA 1.2mM,. Regarding to membrane integrity, treatment MEL 1 μ M showed significantly better in percentages of intact cells (intact plasma membrane, intact acrosome and high mitochondrial membrane potential). Cells with oxidative stress not differ between treatments. Based on the analyzes, it is possible to conclude that the treatment MEL 1 μ M improves sperm membrane integrity in the equine sperm cryopreservation process.