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 -196ºC. 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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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 spermatozoids 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 spermatozoids 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 $60x10^6$ spermatozoids/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 spermatozoids 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 eletroejaculator, were analyzed. The samples were diluted in the means of one fraction BotuBov® (Botupharma®, Botucatu, Brazil) to final concentration of 60x106 sptz / 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 0.25ºC / 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 systemas. 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 carboxiflureseína 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 ± 0,8ºC, and relative humidity of 81.3 ± 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, ºC) was assessed using thermometry and surface temperatures of orbital area (ORB, ºC) and scrotum (SCR, ºC) 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±0.5°C, ORB=36.1±0.8°C, SCR=33.3±1.1ºC, which exhibited significantly 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±6.2% and 21.2±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±1.2ºC, 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±1.0ºC, VPT=32.1±1.0ºC, ATT=32.6±0.9ºC and GRT=5.7±0.9ºC) and T3 (RT=38.4±0.3°C, DPT=32.5±1.8°C, VPT=31.4±2.0ºC, ATT=32.1±1.7ºC and GRT=6.3±1.6ºC). 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±0.63ºC) and T3 (1.1±0.79ºC). 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 $20x10^6$ 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 1µl 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 15ºC

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Keywords: cooling, coloid, 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 15ºC 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 $50x10^6$ sptz/mL. Semen samples were refrigerated for 8 hours at 15ºC 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 ressuspended with the same extender. Progressive motility (%) was evaluated by Computer Asssited 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 ressuspension (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 ressuspension (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 15ºC 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, µm/s VAP – average speed path, µm/s VCL - curvilinear velocity, µm/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 batche was 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 technitian, 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, kinects, 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 39ºC in a pool of seminal plasma (EPP). The three sperm groups were selected in Percoll gradient 45:90% and were incubated at 39ºC for 24 h in SP-TALP buffer medium in a final concentration of $2x10^6$ 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 propidiun 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.

Acknowledgments: FACEPE (APQ-1072-5.04/12), UNIVASF.

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 ($1x10^6$ 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 ± 3.6%, $P < 0.05$). Sperm incubation at 38.5°C affected ($P < 0.05$) sperm motility at different incubation times (68.12 ± 3.6%, 53.75 ± 3.6%, 41.25 ± 3.6% and 27.12 ± 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 ± 3.6%, 43.75 ± 3.6%, 20.62 ± 3.6 % and 3.62 ± 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 spermatic 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 spermatic 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 spermatic 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 spermatic 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-frutose (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 stablish 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 ± 4.99%; VIG, 4.00 ± 0.15 and 3.22 ± 0.19; Pathol, 7.08 ± 0.91 and 6.1 ± 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^{o}C. Finaly, 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^{o}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 $60x10^6$ 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 45º 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 Pecoll 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 1μM, 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 acrossomal 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 1μM and FA 1.2mM,. Regarding to membrane integrity, treatment MEL 1μM 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 1μM improves sperm membrane integrity in the equine sperm cryopreservation process.

A024E Physiology of Reproduction in Male and Semen Technology

Vitrification of epididymal sperm from Iberian ibex (*Capra pyrenaica*)

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Keywords: cryopreservation, Iberian ibex, semen vitrification.

Vitrification, a process in which living cells undergo glasslike solidification, is a relatively new cryopreservation method that can successfully preserve the embryos, oocytes, and even the sperm of certain species. For sperm, at least in the species studied so far, kinetic vitrification would appear to be a better alternative. The simplicity of this sperm cryopreservation technique can be useful in field laboratories for wild species because requires less equipment, is much faster, simpler and cost-effective than conventional freezing. The aim of this work was to evaluate comparatively the effectivity of kinetic vitrification and conventional freezing of epididymal sperm from Iberian Ibex (Capra pyrenaica). Testes were obtained from mature ibexes that were legally hunted in the Tejeda and Almijara Game Reserve, in southern Spain (36ºN latitude, Province of Malaga, Spain) during the rutting season (November/December 2013/2014). Epididymal spermatozoa were collected by the retrograde flushing method, using 1 mL of Tris-citric acid-glucose medium (TCG) at ambient temperature (11-13ºC in the field laboratory). Sperm from left epididymis were frozen with TCG-6% egg yolk and 5% glycerol, and sperm from right epididymis were vitrified with TCG-6% egg yolk with 100 mM sucrose. There weren't differences between treatments (frozen-thawed vs vitrified-warmed sperm) for the percentage of motile sperm, percentage of sperm with membrane integrity determined by the hypo-osmotic swelling test, and percentage of sperm with morphological abnormalities (%). However there were significant differences for quality (score 0-4) of motility (2.4±0.2 and 1.4±0.2), percentage of progressive motility (22.7±4.3 and 7.0±1.6%), percentage of intact acrosome (73.8±4.0 and 55.9±2.5%), percentage of viable sperm according to the nigrosin-eosin staining (45.5±4.1 and 29.2±4.1%), percentage of dead sperm with damaged acrosome (5.5±1.0 and 17.3±2.3%) and percentage of live sperm with intact acrosome (45.1±5.5 and 26.5±4.6) respectively. Although better results were found using the conventional freezing method, given the simplicity of sperm vitrification its use under certain field conditions can be recommended for this type of species. Improvement of the technique might, however, provide better post-vitrification outcomes; new vitrifying solutions and additives should be assessed in future work.

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A025E Physiology of Reproduction in Male and Semen Technology

β-defensin 126 and sperm function in cattle

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Keywords: β-defensin 126, cattle, cauda epididymis, sperm migration.

β-defensins are antimicrobial peptides also thought to have a role in sperm function. In cattle, β-defensin 126 (BD126) has been only detected in the male reproductive tract, with preferentially in the epididymis (Narciandi et al., Immunogenetics 63, 641–651, 2011). The macaque ortholog has been shown to enhance the ability of sperm to migrate through cervical mucus (Tollner et al., Hum. Reprod. 23, 2523–2534, 2008). A mutation in the BD126 gene has been linked to subfertility in men, only explained by reduced ability to penetrate through mucus *in vitro* (Tollner et al., Sci. Transl. Med. 3, 92ra6, 2011). The aim of this study was to examine the role of bovine BD126 in sperm function. Western blot (WB) analysis with a BD126 specific monoclonal antibody demonstrated significant BD126 on bovine sperm which previously published methods for macaque sperm failed to remove. WB analysis also revealed that while BD126 is present on sperm and in seminal plasma from intact bulls, it is undetectable in the ejaculate of vasectomised animals, indicating that it does not originate in the accessory glands. Further analysis demonstrated that the peptide is uniquely present in the cauda epididymis and is absent from sperm recovered from other epididymal regions, thus providing a model to study its function. Confocal analysis revealed immunofluorescent labelling of BD126 specific to the tail and acrosomal region in cauda sperm only, suggesting a role in sperm motility. We therefore hypothesized that addition of cauda fluid to corpus sperm would improve motility and ability to penetrate cervical mucus *in vitro*, and that this may be due to the activity of BD126. Testes were collected from adult bulls at an abattoir and sperm from the corpus and cauda epididymis, as well as cauda epididymal fluid (CEF), were recovered. Corpus sperm were incubated for 1 h with CEF in the absence or presence of BD126 antibody (Ab); untreated corpus and cauda sperm were used as controls. A higher number of cauda than corpus sperm migrated through cervical mucus ($P<0.001$) and addition of CEF increased the number of corpus sperm migrating through this matrix ($P<0.05$). The presence of the BD126 Ab failed to abrogate this effect. Analysis of motility using a computer assisted sperm analysis system indicated higher total and progressive motility in caudal sperm when compared with sperm from the cauda ($P<0.001$); again, addition of CEF increased progressive motility ($P<0.05$). In conclusion, we have characterised the expression of bovine BD126 as a protein in the cauda epididymis. Incubation of sperm from the corpus epididymis (which lack BD126) with CEF from the cauda (which contains BD126) resulted in enhanced sperm migration through cervical mucus, and higher motility. Further work will clarify the role of BBD126 and related β-defensins in mediating bovine sperm function.

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A026E Physiology of Reproduction in Male and Semen Technology

Expression of β- nerve growth factor in rabbit male tract and seminal plasma

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Keywords: β-NGF, male reproductive tract, seminal plasma, rabbit

Nerve growth factor (NGF) has been recently identified as an ovulation inductor factor (OIF) in the seminal plasma (SP) (Ratto et al. PNAS 2012; 109:15042-7). The presence of OIF in rabbit has been suggested but this protein has not yet been identified. Our aim was to study the mRNA expression in the rabbit male reproductive tract and to identify the protein β-NGF in the SP. Total RNA was extracted from prostate, testicles and seminal glands of 3 male rabbits (TRIzol® Plus RNA Purification Kit, Life Technologies) to subsequently isolate mRNA (FastTrack® MAG mRNA Isolation Kit, Ambion, Life Technologies,) for retrotranscription to generate cDNA. Specific primers were designed on the mRNA sequence deposited in GenBank (XM_008264614.1) to target a highly conserved region of NGF among species (5'-AGCCCACTGGACTAAACTGCA-3';5'-TCGCACACCGAGAACTCTCC-3'; product size: 305 nucleotides). PCR was performed on cDNA to obtain the expected 300 pb fragment that was sequenced confirming the presence of NGF-mRNA in seminal plasma, testicle and prostate. To determine the expression of mature NGF protein in SP, an aliquot was prepared from collected semen, centrifuged at 3000xg for 30 min at 4ºC and stored at -20°C. For Western blot (WB) analysis, samples were loaded in 12% SDS–PAGE and electrotransferred onto nitrocellulose membranes. The membranes were probed with mouse β-NGF antibody (Promega) using donkey anti-mouse as secondary antibody (Li.Cor Biotechnology). Blots were scanned in an Odissey Infrared imaging system. In addition, NGF was purified by offgel technique with the 3100 OFFGEL Kit pH 3–10 (Agilent Technologies Inc) and the recovered fraction recognized with the mouse β-NGF antibody was used for mass spectrometry analysis (MS) (4800 Plus Proteomics Analyzer Applied Biosystems,). MS was operated in positive reflector mode with an accelerating voltage of 20,000 V. For protein identification NCBInr was used. Database without taxonomy restriction and a home-made database with the sequence of NGF (gi|655847230) downloaded from NCBInr was searched using MASCOT v 2.3. The probability scores of NGF sequences from several species were greater than the score fixed by MASCOT as significant with a p-value < 0.05. Our results show that expression of NGF-mRNA were clearly identified in the rabbit male tract organs above described and the corresponding mature protein band with a mass of ~60 kDa was also identified by WB whereas a ~13 kDa band was detected in the basic fraction (pH=8.24-8.83) obtained when offgel electrophoresis was performed. Furthermore, protein identification by mass spectrometry revealed the existence of NGF in the SP. In conclusion, mRNA and protein NGF are present in rabbit male reproductive tissue and SP respectively, providing the basis to undertake further functional analysis for its potential role in rabbit reproduction.

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A027E Physiology of Reproduction in Male and Semen Technology

Assessment of bull semen quality loaded in new SensiTemp straws using semen and IVP technologies

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Keywords: bull semen, fertilization, IVP, SensiTemp straws.

SensiTemp, a new IMV bull straw concept, presents the advantage of color changing while the straw is thawed. The color of frozen straws is blue and straws start to become white when the temperature reaches 33°C, with a complete change of color at 37°C. The objective of this study is to assess quality after thawing of semen frozen in SensiTemp, *in vitro*, using Computer Assisted Semen Analysis (CASA), Flow Cytometry (FC) and *In Vitro* Fertilization (IVF). The ejaculates of two bulls, selected during preliminary experiments on high *in vitro* fertility, were harvested at CIA L'Aigle, France and split ejaculates were frozen in experimental (SensiTemp) and conventional (Control) straws. In experiment 1 after thawing semen from the two type of straws (*5 pooled straws each; 2 replicates*), motility was assessed using the IVOS CASA system (Hamilton Thorne Inc., Beverly, MA, USA) and membrane integrity was evaluated through FC with Cytosoft software (Millipore-Guava Technologies Inc., Hayward, CA). In experiment 2, IVF was used to evaluate the non toxicity of SensiTemp and control straws. Cumulus-oocyte complexes (COC; *n=1178; 4 replicates*) collected from slaughterhouse ovaries were matured in IVM medium (TCM-199 with bicarbonate, Sigma-Aldrich, Saint Quentin Fallavier, France; 10µg/ml FSH-LH, Reprobiol, Liège, Belgium and 10% FCS, Thermo Fisher, Illkirch, France) for 22 h. After fertilization, presumptive zygotes of each group (SensiTemp and control for each bull) were cultured in synthetic oviduct fluid medium (SOF, Minitube, Tiefenbach, Germany) with 1% ECS and 0.6% BSA (Sigma-Aldrich, France) up to 8 days. All cultures were conducted at 38.5C in 5%CO2, 5%O2. The cleavage and blastocysts rates were evaluated on Day 3 and 7, respectively for each group. Embryo quality was recorded on day 7 according to the IETS evaluation. Data from each bull were analyzed separately using the Chi square test ($P<0.05$). In experiment 1, neither sperm motility from bull 1 (61.2 and 60.5%) and bull 2 (66.2 and 66.5%) nor membrane integrity from bull 1 (58.6 and 52.2%) and bull 2 (61.0 and 61.9%) were different between SensiTemp and Control, respectively. Results from experiment 2 showed no difference ($P>0.05$) in cleavage rate between SensiTemp and Control for the two bulls: 92.1 and 91.7% for bull 1 and 94.2 and 94.6% for bull 2 respectively. The blastocysts rate on day 7 did not differ ($P>0.05$) among groups (47.5, 47.1 and 51.3, 50.4% for SensiTemp and Control bull 1 and bull 2, respectively) nor the quality of embryos retrieved in the different groups: 25.4, 23.3 and 30.8, 29.6% in grade 1 embryo for SensiTemp and Control bull 1 and bull 2, respectively. Those results demonstrate, *in vitro*, that the new SensiTemp straws were non toxic and did not affect the semen quality after thawing nor did the SensiTemp straws affect the ability of sperm cells to fertilize oocytes and produce 8 days old embryos.

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A028E Physiology of Reproduction in Male and Semen Technology

Hyaluronic acid-binding ability of spermatozoa and its role for selection of vacuole free human spermatozoa in human reproduction

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Keywords: ART outcomes, DNA integrity, sperm head vacuoles, MSOME, sperm selection, hyaluronic acid binding.

The type of spermatozoa selected in ART (assisted reproductive technology) influences the outcome in regard to embryo development, pregnancy, miscarriage and malformation. Sperm head nuclear abnormalities were identified earlier as vacuoles by motile-sperm organelle-morphology examination (MSOME). Blastocyst development and the pregnancy rates are negatively influenced if vacuoles containing sperm are used for ICSI. Thus, it is of importance to reliably select vacuole-free spermatozoa in assisted reproduction. In a prospective, observer blinded study. Hyaluronic acid (HA) bound, standard morphological (SM) selected (200x) and unselected sperm were collected by different examiners. The evaluation of vacuoles by Nomarski differential interference contrast (DIC; 600x up to 7.200x) was performed observer blinded for all samples. Eleven human semen samples were prepared by a 80% density gradient. From each sample a minimum of 20 sperm per method (HA and SM selection) were collected in separate PVP droplets. Additionally, 20 unselected spermatozoa were collected from each sample designated as control. The number of vacuoles in each sperm head was determined by means of DIC. One way analysis of variance was performed (Tukey-Test; Sigma Stat Version 3.5, DUNDAS Software LTD.). Significantly more sperm without vacuoles were found in HA selected ($p<0.001$) and SM selected ($p<0.001$) than in unselected samples. The number of sperm with one or two vacuoles ($p<0.01$) and more than two vacuoles ($p<0.001$) was significantly higher in the unselected group. Furthermore, in HA selected sperm the appearance of two vacuoles was significant lower than in SM selected sperm ($p<0.05$). Both selection methods provide spermatozoa containing less vacuoles than in the unselected samples, especially in the group with more than two vacuoles. This shows that HA selection is a good method to select spermatozoa in regard to the appearance of vacuoles. This is of significance since the HA selected spermatozoa are more mature, with less cytoplasmatic retention and higher DNA integrity than unselected sperm cells. Thus HA selection may be an effective method to identify spermatozoa with a higher potential in reproduction in order to improve safety and results in ART procedures.

A029E Physiology of Reproduction in Male and Semen Technology

Seasonal variation of testicular functionality in alpaca (*Vicugna pacos*) raised in Italy

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Keywords: alpaca, scrotal edema, testicular measures, ultrasonography.

Thermoregulatory functions of testicles are very important for sperm viability in terms of spermatogenesis and maturation phases. Unique characteristic are present in South American camelids related to the position, body mass/testicle volume ratio and anatomical features (epididymis orientation). Among different parameters to evaluate male there are testicular dimension measures (width, length and thickness). It has been observed different times the increase of testicular volume during heat season in animals with pendulous testicles because of circulatory impairment. Aim of this study was to monitoring physical and physics parameters besides the semen quality evaluation during two different seasons (summer, winter) in alpacas. Eight adult males are evaluated considering classical (testicular measures – caliper measurements) and innovative parameters as ultrasonography of the testicles. Semen collections were performed with a teaser and ejaculates obtained were destined to the quality assessment (volume, colour, viscosity, motility and concentration) and biochemical evaluation of the seminal plasma (energetic, protein and enzymatic profile – Hitachi 912 biochemical auto-analyzer). Data were analysed for ONE-WAY ANOVA considering the season as variable independent and the parameters evaluated as dependent variables using the statistical software SIGMASTAT 2.05. There was a significant difference among seasons with a general decrease of the semen quality during the hot season. The lower levels of volume, concentration, seminal plasma (SP) glucose, SP cholesterol, SP triglyceride, SP Phosphates and the higher levels of SP Gamma Glutamil Transferase, SP Alkaline Phosphatase, SP Magnesiumn clearly indicate a detrimental effect of high environmental temperature because the effect on testicular thermoregulatory capability. Negative correlation between Testicular Measures and semen quality parameters was significant (r: -0.64 - -0.45) . At the ultrasound evaluation was characterized the reason of increased testicular mass during the hot season considering the evidence of scrotal edema. The scrotal edema derived by a defect of local circulatory mechanism. Testicular functionality may be influenced by the high environmental temperature and specifically in alpaca were the position of the gonads imposes a fine regulatory pattern. Hot season causes a testicular circulatory defect with a scrotal edema as results and a decrease of semen quality.

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A030E Physiology of Reproduction in Male and Semen Technology

Characterization of accessory glands ultrasonography in rams of endangered venetian sheep breeds

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Keywords: accessory glands, endangered Venetian Sheep Breeds, breeding soundness evaluation, ultrasonography.

Evaluation of male needs standardized protocol for the male's classification. Breeding soundness evaluation (BSE) is a practice that is widely used, mainly in the bull, to evaluate the male starting from physical and reproductive parameters. BSE protocols for rams are already published around the world but complete evaluation is not yet raised for all the breeds with specific characteristics. The male can be classified as Satisfactory, Questionable and Unsatisfactory. When is not possible to evaluate it properly, the classification can be deferred. Among the innovative methods to perform the evaluation, there is the ultrasound exam of the reproductive organs. Testicles, epididymis, vascular cone and accessory glands ultrasound may increase the accuracy of the evaluation. In this study, an established sample of rams belonging to Veneto Agricoltura Center in Villiago (BL), Italy, has been evaluated with classical and innovative monitoring system. On these animals the entire BSE procedure was carried out. Moreover, the ultrasound evaluation (MyLabVet^{TM} One, ESAOTE S.p.A., Genova, 10 MhZ probe frequency) of testicles and vesicular glands has been performed for the first time in these breeds (18 adult rams: N=5 Brogna, N=5 Lamon, N=4 Foza, N=4 Alpagota). After the physical and physics exams all the males involved in the evaluation were collected using electro-ejaculator (Ruakura Ram Probe Plastic Products, Hamilton, New Zeland); the trans-rectal probe was inserted after a mucosal anesthesia (5 ml of Lidocaine 2 %) performed during the deferent ampullas massage. Procedure of semen quality evaluation considering general ejaculate parameters (color, volume, concentration) and specific microscopic observation about viability fresh-post thawed with differential staining (Eosin/Nigrosin, Spermac and Farrelly staining), kinetic CASA parameters (Ivos II, Hamilton Thorne, Germany). Data analysis (Pearson correlation indices) revealed important correlations among scrotal circumference, serum testosterone and semen kinetic parameters. Furthermore, increasing the testicular parenchyma echogenicity, the semen volume used to lower. Testicular and vesicular glands ultrasound exam give us important information about seminal plasma quantity. Particularly vesicular glands echogenicity has shown high relationship with quantity of seminal plasma and therefore low sperm concentration. Physics equipment as ultrasonography may optimize collection procedure performed with electro-ejaculator. Body mass and vesicular glands dimension can influence the induction success and the semen freezeability.

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A031E Physiology of Reproduction in Male and Semen Technology

The joint treatment of sperm by prolactine and GTP have determined the increase of the number acrosome-reacted spermatozoa in bulls

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There are contradictory opinions concerning involvement of prolactin (PRL) in the process of sperm capacitation and acrosome reaction (Vigil P. et al., 2011 Biol Res, 44:151-159). It was shown that PRL stimulates release of Ca^{2+} from IP3-sensitive stores, and GTP stimulates release of this ion from IP3-insensitive stores (Denisenko V. et al., 2015 Tsitologiya, 3:1-8). GTP forms a connection between IP3-sensitive and IP3-insensitive intracellular stores and promotes transition of Ca^{2+} between these stores (Mullaney J. et al., 1987 J. Biol. Chem. 262: 13865—13872). The aim of the present study was to examine the mobilization of Ca^{2+} from intracellular stores after the treatment spermatozoa by PRL and GTP and to evaluate the status of spermatozoa after these treatments. Intensity of fluorescence of membrane-bound Ca^{2+} was determined with a fluorescence spectrophotometer Hitachi MPF-4 (excitation: 380-400nm, emission: 530 nm) using 40 μM chlortetracycline (CTC) - (Denisenko V. et al., Tsitologiya 3:1-8, 2015). Intensity of fluorescence of membrane-bound Ca^{2+} was determined in Sp-TALP medium where the concentration of cells was adjusted to 1, 5 X 10^6 sperm/mL. The CTC assay was used to determine the functional status of spermatozoa (Ded L. et al., 2010 Reprod Biol Endocrinol, 8-87). Samples were examined with fluorescence microscope *Zeiss Axo Imager* M1. Ejaculates from three fertile bulls were used, and five replicates were performed for each experiment. In each sample, 200 cells were evaluated. Sperm were evaluated according to 1 of 3 CTC staining patterns: fluorescence over the entire head (precapacitated cells), fluorescence-free band in the postacrosomal region (capacitated cells) and low fluorescence over the entire head except for a thin bright fluorescent band along the equatorial segment (acrosome-reacted cells). All reagents that were used in this study were produced by Sigma-Aldrich (Moscow, Russia). Data were analyzed by Student's t-test. Treatment spermatozoa by PRL (10 ng/ml) or GTP (10 μmol) resulted in release of Ca^{2+} from intracellular stores (0.70±0.019 and 0.69±0.017 vs 0.85±0.016; $P<0.001$). There was additional release of Ca^{2+} with the combined effect of PRL and GTP (0.62±0.011 *vs* 0.70±0.019 and 0.69±0.017; $P<0.001$). There was no additional release of Ca^{2+} after the joint action by the pair of these reagents in the presence of protein kinase C inhibitor (Ro 31-8220, 10ng/ml). The average percentages of capacitated spermatozoa did not change after treatment by PRL, GTP or both these reagents. The percentage of cells that underwent acrosome reaction have increased after treatment by PRL and GTP jointly (46% *vs* 62%, $P<0.01$); there was no such effect at preliminary treatment of sperm by Ro 31-8220 (10 ng/ml). Thus, Ca^{2+} transition between intracellular stores in bull spermatozoa after the treatment with PRL and GTP jointly is leading to increasing in the percentage of acrosome-reacted spermatozoa.