A001 Male Reproductive Physiology and Semen Technology

# Bovine cyropreserved epididymal spermatozoa viability after incubation by different periods of time in IVF medium

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**Keywords:** epididymis, sperm quality, heparin.

The recovery of epididymal spermatozoa, their cryopreservation and posterior use on IVP of bovine embryos, has become an important tool to store genetic material from animals that have died unexpectedly or that have acquired reproductive inability. The present study aimed to evaluate the viability of spermatozoa from epididymides after their incubation on IVF medium supplemented or not with heparin. Spermatozoa were recovered from epididymis of bulls (n=3) and were cryopreserved. In each replicate one straw from each animal was thawed to form a pool, which was centrifuged in percoll 45% at 700g for 10 minutes. After centrifugation, one sperm sample was taken (0h) to assess total and progressive motility (CASA), morphology (phase contrast), capacitation (chlortetracycline-CTC), membrane integrity (6-carboxy-fluorescein diacetate and propidium iodide-FDA-IP) and acrosome integrity (isothiocyanate-conjugated peanut agglutinin and PI, iodide de propídeo-PNA-FITC-IP). The remaining sample was incubated in IVF medium with or without heparin (hep=10μg/ml) for 3, 6 e 9 h (3h-hep, 3h+hep, 6h-hep, 6h+hep, 9h-hep and 9h+hep, respectively). Three replicates were performed and data were analyzed byANOVA and Tukey test ($P<0.05$). No differences were detected among groups regarding morphology (44.7% to 61.7%), capacitated cells (15.3% to 26.4%) and intact acrosome (21.5% to 33.6%). The percentage of cells with intact membrane was similar between the groups 0h (43.8±6.6%), 3h-hep (33.4±4.8%), 3h+hep (31.5±8.0%) and 6h-hep (31.5±8.05%). However, group 6h+hep (26.5±4.4%) showed a lower percentage compared to group 0h, but was similar to groups 9h-hep (22.5±5.3%) and 9h+hep (26.5±7.9%). Compared to 0h group (75.3±14.0%), a decreased in total motility was observed at 6 h of culture (6h-hep=35.7±8.4%, 6h+hep=45.0±4.0%), which was similar to that observed at 9 h with (30.0±4.9%) or without heparin (34.7±11.2%). A significant decrease in progressive motility was only observed at 9h of culture (0h=39.0±10.8%, 9h-hep=23.3±9.2% e 9h+hep=22.7±10.1%), and no effect of heparin was also detected for this parameter. It can be concluded that spermatozoa from epididymides, when cultured in IVF medium, maintained their viability characteristics unchanged in the first hours of culture, regardless the presence of heparin. In addition, up to 9 h of culture only motility and membrane integrity values were altered.

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# Use of 2',7'dichlorofluorescein dicacetate staining in ram sperm cells for oxidative stress detection

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**Keywords:** flow cytometry, oxidative stress, spermatozoa.

The reactive oxygen species (ROS) act in several physiologic processes, such as capacitation and sperm hyperactivation. However, when there is non-equilibrium between ROS and antioxidants, it can cause oxidation of biomolecules, known as oxidative stress. Oxidative stress, in the spermatozoa, can affect semen quality, modify motility and mitochondrial activity, and generate lesions in DNA (Andrade, E.R, Ver. Bras. Reprod. Anim., v.34, n.2, p. 79-85, 2010). The dichlorofluorescein is a marker for intracellular hydrogen peroxide. This tool has already been used for evaluating humans, porcine and canine semen (Mahfouz,R., Fertility and Sterility, v.92, n.2, p.819-827, 2009, King, S.,Anim. Rep. Sci., 119, p. 106-114, 2010, Aziz, N., Fertility and Sterility, v. 94, n.7, p. 2604-2608, 2010), however has not been yet elucidated for ram sperm. The objective of this study was to evaluate the use of 2 ', 7' dichlorofluorescein diacetate by capillary cytometry (CC) for oxidative stress analysis in ram semen. For this purpose, 12 adult, 7 months old rams, were evaluated. Semen was collected by artificial vagina weekly, over four weeks. The samples were evaluated for progressive motility and spermatozoa concentration. The final dilution of 4,000 cells/µL, it was done in TL-semen. The samples diluted were incubated in 3.5 µL of 2 ', 7' dichlorofluorescein diacetate (DCF) and 0.5 µL of propidium iodide (PI) for 5 minutes. PI was used to exclude cells with membrane damage, non-viable in the evaluation of DCF. Sample suspension was achieved by adding phosphate buffer solution (PBS) without Calcium and without Magnesium. The reading was performed by CC (Guava Easy Cyte Mini) and measured between 500 and 530nm spectrum for green fluorescence. The statistical analysis was performed using analysis of variance comparing the four weeks and a comparison test of means (Tukey) at a 5% level. The mean and standard errors of positive cells for DCF and negative cells for DCF was respectively, 10.6 ± 5.0 and 89.4 ± 5.0 in the first week, 9.4 ± 2.2 and 90.7 ± 2.2 in the second week, 15.5 ± 1.1 and 94.8 ± 1.1 in the third week and fourth week 5.9 ± 1.0 and 90.1 ± 4.0. No difference was observed ($p>0.05$) between weeks. It is concluded that the use of dichlorofluorescein by CC to assess the presence of reactive oxygen species in ram semen is effective.

A003 Male Reproductive Physiology and Semen Technology

# Influence of different spermatozoa selection methods on acrosomal integrity, viability and other spermatic parameters of goat frozen-thawed semen

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**Keywords:** goat, sperm selection, trypan blue/giemsa.

The freeze-thawing semen procedure causes several spermatozoa damages. Sperm selection has an important role to obtain viable spermatozoa at elevated concentration for in vitro fertilization. This study aimed to evaluate the effects of three spermatozoa selection methods on different spermatic parameters of goats. Nine frozen-thawed semen samples from Saanem goats with age between two and five years were used. Three spermatic selections methods were tested: swin-up (SWU) or ascendant migration, Percoll density gradient (GP) and sperm wash (LC). All samples were evaluated in triplicate. The motility, vigor and spermatic concentration parameters were analyzed after and before the selection methods. The spermatic recovery rate, viability and acrosomal integrity were evaluated by Trypan Blue/Giemsa staining. The results were analyzed by ANOVA and means compared by Student Newman Keuls. The values are presented as mean±SEM. There was no difference ($P>0.05$) among males for motility and viability parameters. The GP recovered more ($P<0.05$) spermatozoa (28.1±3.3%) and with greater motility (55.5±2.6%) than SWU (2.27±0.3% and 41.1±5.0%, respectively), but the spermatic recovery rate was lower ($P<0.05$) than LC (43.3±3.8%). There was no difference ($P>0.05$) among the three treatments for viability (51.3±4.0%, 47.5±2.3% and 47.1±3.3% for SWU, GP and LC, respectively), but the proportion of live spermatozoa without acrosome was higher ($P<0.05$) for SWU than the other methods (4.0±0.8%, 1.8±0.3% and 2.5±0.4% at SWU, GP and LC, respectively). These results show that GP and LC methods increase the recovery of spermatozoa from frozen-thawed semen with better viability and lower acrosome loss.

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A004 Male Reproductive Physiology and Semen Technology

# Reduction in force of mini percoll centrifugation decreases the formation of reactive oxygen species (ROS) in bovine semen

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**Keywords:** bovine semen, centrifugation, reactive oxygen species.

Several methods have been used in the selection of bovine spermatozoa for in vitro fertilization (IVF), and the Percoll gradient technique is the most commonly used. In order to improve the recovery of sperm and serve a market in constant expansion like IVF, and from sexed semen, changes in the force of centrifugation have been applied without previous analysis. Our goal in this study was to evaluate the effect of different centrifugation forces in the process of sperm selection by Mini Percoll on the sperm recovery and viability. Five replicates were evaluated, using a pool containing semen from four bulls Bos taurus, distributed in four treatments: T1 (9000G Control), T2 (6700G), T3 (4500G) and T4 (2200G). The straws were thawed at 35°C for 20", homogenized, and the semen deposited on 1.5 ml tubes each containing 300ul Percoll gradient (90, 60 and 30%) and centrifuged for 5 ', according to the treatment. The pellet formed was resuspended in 300ul of FERT-TALP medium and centrifuged for 1' at 9000G, and 100ul of the final pellet for assessment of viability. The criteria used for evaluation of sperm viability were motility, force, concentration, morphology, membrane integrity and reactive oxygen species (ROS) generation. To evaluate motility, concentration, membrane integrity and spermatozoa morphology a phase contrast microscopy was used. ROS levels in semen were determined by spectrofluorimetric method using 2', 7'-dichlorofluorescein diacetate (HD-D). Data were analyzed by chi square ($X^2$) and ANOVA, the means were compared by Tukey and Duncan test at 5% of significance. The values of membrane integrity, concentration, force and sperm pathology did not differ between treatments. The motility of T3 and T4 (87% and 83%) was higher than T1 (67%), but did not differ from T2 (71%). Increased formation of ROS was observed in T1 (61) compared to T2 (44), T3 (42) and T4 (46), which did not differentiate between them and suggest that the reduction of centrifugation force has provided greater preservation of antioxidant mechanisms of semen, showing a smaller formation of ROS. Based on these results, we conclude that the centrifugation forces tested did not affect sperm recovery, with greater sperm viability in the T2, T3 and T4, which generated less ROS. Other studies should be conducted in order to evaluate the influence of centrifugal force reduction in fertilizing capacity and embryo formation.

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# Correlations between testicular vascularity by color doppler ultrasound and sperm characteristics in rams

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**Keywords:** ovine, hemodynamic, spermatozoa.

The color Doppler ultrasonographic mode exam provides images of blood flow of the tissue simultaneously to the gray scale morphology (B-mode), being applicable to the evaluation of tissue vascularization. Therefore, the aim of this study was to evaluate the hemodynamic of ram testis and correlate the data with the semen quality. Seven adult rams with different reproductive histories were used, varying from normal testes to various degrees of degeneration. The rams were evaluated weekly during two month for body temperature, testicular temperature, respiratory rate, testicular consistency, scrotal circumference, parenchyma and the pampiniform plexus ultrasonography and semen characteristics (volume, concentration, whirling, motility, vigor and sperm morphology). The ambient temperature was measured weekly. The ultrasonographic exam of the testicular parenchyma was performed in the two-dimensional mode (B-mode), classified on scales from 0 to 3 for the presence of hyperechoic spots (calcifications) and in the color Doppler mode, being classified in scores from 1 to 4, from the lower to the higher intensity of vascularization. After that, we performed the analysis of the pampiniform plexus at color Doppler mode (scores 1 to 4) and spectral mode, to evaluate the resistance index (RI) of the testicular artery. The classification of the scores was made with double blind test. The results were analyzed employing the Statistical Analysis System (SAS Institute Inc., 1995) being checked for normality of residuals by the Shapiro-Wilk test (Proc Univariate). The data that did not meet statistical assumptions were subjected to logarithmic transformation [log (X +1)] and were analyzed using Pearson correlation test, being the significance level of 5%. Positive correlations were observed between the respiratory rate and: rectal temperature ($r = 0.44$, $p < 0.0001$), testicular temperature ($r = 0.38$, $p = 0.0003$), ambient temperature ($r = 0.31$, $p = 0.0037$), pampiniform plexus score ($r = 0.37$, $p = 0.0004$) and RI ($r = 0.24$, $p = 0.0248$). RI was positively correlated with rectal temperature ($r = 0.41$, $p < 0.0001$) and motility ($r = 0.32$, $p = 0.0027$) and negatively correlated with minor defects ($r = -0.33$, $p = 0.0023$), plexus pampiniform score ($r = -0.30$, $p = 0.0047$) and total of sperm defects ($r = -0.28$, $p = 0.0100$). Positive correlation was found between plexus pamipiniform score and parenchymal calcifications score ($r = 0.43$, $p < 0.0001$). Sperm motility was positively correlated with vigor ($r = 0.87$, $p < 0.0001$) and whirling ($r = 0.66$, $p < 0.0001$) and negative correlated to total sperm defects ($r = -0.33$ $p = 0.0018$). The study demonstrated the existence of correlations among several parameters such as temperature, seminal characteristics and vascular features of the testes. However, more studies are still needed in this aspect.

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A006 Male Reproductive Physiology and Semen Technology

# Parameters obtained in the andrologycal examination in bonsmara bulls

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**Keywords:** andrologic evaluation by points, *Bos taurus taurus*, semen.

The Bonsmara breed was developed in 1937 by the South Africa Department of Agriculture. In Brazil, it was introduced in 1997, through the importation of semen.The objective of this study was to report the parameters obtained in the andrological examination in Bonsmara bulls. The animals were examined in September 2011, in a property used for raising cattle beef, located in Paraguaçu Paulista city, São Paulo, Brazil. Thirty-nine Bonsmara bulls breed (Bos taurus taurus), raised extensively, had ages ranging from 33 to 74 months old (45.3 ± 8.8 ), were submitted to andrological examination and andrologic evaluation by points (PAC) (Vale Filho,V.R. In: Congresso Brasileiro de Reprodução Animal, 8, Anais. p.94-118). The animals were were clinically healthy, with average body condition score 3 (scale of 1 to 5). For the examination of the external genitalia, has performed the inspection and palpation, being assessed the scrotum, testicles, epididymis, sperm, foreskin and penis. In internal examination, vesicular glands, phial? of the deferent duct, prostate and bulbouretral gland were examined by transrectal palpation. Measurements of scrotal circumference were performed with the aid of measuring device, positioned at the middle region of the scrotum, in the area with the largest diameter, involving the two gonads and the scrotum. The electroejaculation was performed to collect the semen on graduated tubes, coupled to plastic funnels. Immediately after collection, were performed evaluations of volume (ml). For assessment of whirling, a drop of semen, placed on a slide preheated to 37 °C, was displayed in optical microscopy with an increase of 100 times. Subsequently, a coverslip was placed also pre-heated to 37 °C on the drop of semen and, with an increase of 400 times to evaluate the motility and force spermatic. The sperm concentration was estimated, using as a base the appearance of the semen in aqueous (<300x106/mm$^3$), opalescent glass (300 to 500x106/mm$^3$), milky (500 the 1.000X106 /mm$^3$) and creamy (>1.000X106/mm$^3$). The bulls were classified regarding their reproductive potential ranking second PAC. The data were submitted to the descriptive statistical analysis and the results were presented as mean and standard deviation. The scrotal circumference was 40.9 ± 3.0 cm. For the sperm motility, the average value obtained in this work was 63.0 ± 23.4 % and force of 2.92 ± 0.98 (0-5). Keepthe showed average value of 1.82 ± 1.83 (0-5). The bulls had a mean of 4.25 ± 4.29 % for sperm defects. The average obtained for total sperm defects was 21.10 ± 12.49 %. The bulls showed 78.8 ± 18.1 (0-100) points, considered as satisfactory score in PAC. In view of the reduced number of publications with Bonsmara bulls breed, it becomes relevant to generate data of andrological examination of bulls kept under different management conditions.

A007 Male Reproductive Physiology and Semen Technology

# Association of breeding soundness evaluation with hormonal and semen protein parameters during the peripubertal period in dairy Gyr bulls

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**Keywords:** IGF-I, insulin, leptin.

Given the importance of andrologic characteristics, especially those related to scrotal circumference (SC) and semen quality, different scoring systems of bulls reproductive performance have been proposed that serve as more appropriate indexes for checking breeding soundness evaluation. The bulls can be evaluated and submitted to table systems of andrologic evaluation by points (CAP) which yield ratings of their reproductive potential. This study was carried out to evaluate breeding soundness (CAP) in precocious and regular Gyr dairy bulls during peripuberty and its relationship with the hormone and seminal protein recorded in this period. The ranking of CAP was performed according to scrotal circumference and sperm condition. The hormone levels in seminal plasma of IGF-I, leptin and insulin were obtained by RIA and the values of seminal protein bands by one-dimensional electrophoresis. Whereas the CAP of precocious animals 23.6 ± 7.3, 28.8 ± 8.9, 34.5 ± 16.2 34.5 ± 16.2, 48.16 ± 12.4 and 66.5 ± 14.1 for -60, -30, 0, 30 and 60 days of puberty, respectively. The CAP values of regular animals were values of 26.0 ± 9.9, 32.0 ± 12.0, 39.8 ± 12.9, 57.3 ± 10.8 and 76.8 ± 14 .6, respectively. No difference ($p>0.05$) was found between groups. The CAP showed a positive correlation of high magnitude with the following variables: age (r=0,6), scrotal circumference (r=0,81), live weight (r=0.8), sperm concentration (r=0,7) and total number of normal cells (r=0.81) ($p<0.001$). Likewise, correlations with the seminal protein bands of 112 (r=0.3), 55 (r=-0.4), 47 (r=-0.7), 27 (r=0.4), 25 (r=-0.8), 22 (r=0.5), 18 (r=0.9), 12 (r=0.5) and 6.9kDa (r=0.4), and the concentrations of IGF-I (r=0.4, $p<0.003$), the protein peaks with affinity to heparin and total protein (r=0.5) were also found. These results suggest the important role that CAP may play in the classification of animals with high capacity for reproduction and andrological standard, linked to factors like protein, humoral factors of local action and weight.

A008 Male Reproductive Physiology and Semen Technology

# Electrophoretic profile of seminal plasma of dairy Gyr bulls in peripuberty and its correlation with sperm parameters

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**Keywords:** puberty, sds-page, sperm, zebu.

The aim of this study was the evaluation of the protein profile of semen, in SDS-PAGE, of Dairy Gyr bulls at peripuberty (60 days before and 60 days after puberty's beginning). This parameter was correlated to sperm parameters and to the protein peaks of heparin binding. Sixteen young bulls were and they were separated in two groups, precocious and regulars, according to its puberty. 1D gels were prepared in a gradient of 10 to 15% of acrylamide, and later evaluated using the program QuantityOne® version 4.6.3. The proteins with affinity to heparin were separated by filtration gel, using quick performance liquid chromatography in affinity columns. Andrologic data was determined according to the Brazilian College of Animal Reproduction's criteria. Precocious animals showed total protein concentrations in the seminal plasma of 13.3±7.7, 12.7±9.6, 13.5±13.4, 37.1±36.1 and 32.8±14.7 mg/mL, respectively, for the time periods of -60, -30, 0, 30 and 60 from puberty, while regular animals showed concentrations of 15.1±12.8, 15.4±8.4, 36.9±24.3, 29.6±17.9 and 34.0±13.7 mg/mL, for the same period, with statistical difference ($p<0.05$) only in the time gap within the groups. Both groups had mean of 22 protein bands in the studied period with molecular weight varying from 238 to 6.9kDa. From the proteins found in the seminal plasma, only the 112, 62, 55, 30, 22, 19, 16, 13.9, 12, 11 and 6.9kDa showed statistical difference within peribuperty period ($p<0.05$), but not between groups. The intensity of 112, 55 and 47kDa bands was correlated ($p<0.05$) to sperm concentration and motility, with values of 0.32 and 0.37, -0.32 and 0 and -0.54 and -0.58, respectively. Scrotal circumference showed positive correlation with the bands 134 ($r=-0.59$), 55 ($r=-0.41$) and 47kDa ($r=-0.73$) and positive correlation to the 112kDa band ($r=0.27$) ($p<0.05$). The peaks of heparin affinity 3, 4, 5, 6, 7 and 8 had negative correlations with 55 and 47kDa bands, showing values of 0.4 and 0.5, 0.3 and 0.8, 0.5 and 0.6 and 0.5 and 0.5, 0.5 and 0.6, 0.2 and 0.6, respectively ($p<0.05$). The proteins with lower molecular weight (<22kDa) can be highlighted as the ones that showed higher concentration and represented 69.8% of the total. They were also positively related to body weight ($r=0.6$), scrotal circumference ($r=0.4$), sperm concentration ($r=0.5$) and motility ($r=0.3$) and negatively related to major sperm defects ($r=-0.6$) and total ($r=-0.6$), also with positive correlation to the peaks 3 ($r=0.4$), 4 ($r=0.3$), 5 ($r=0.5$), 6 ($r=0.6$), 7 ($r=0.6$) and 8 ($r=0.4$) of heparin affinity ($p<0.05$). The seminal protein profile of Dairy Gyr bulls differed only within age, suggesting the proteins of 55 and 47kDa as negative markers and the proteins with molecular weight <22kDa as positive markers for seminal quality and apparently no important to sexual precocity.

A009 Male Reproductive Physiology and Semen Technology

# Effect of spermatic selection with Equipure® on the refrigeration of stallions semen

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**Keywords:** cooled semen, equipure, stallion.

The spermatic selection using the density gradients aims to improve the quality and length of viability of semen, once it selects the sperm with progressive motility and normal morphology from the ejaculated. The objective of this study was verify the effect of the spermatic selection using Equipure® (Nidacon, Botupharma, Botucatu-SP, Brazil) on the spermatic kinetics and longevity of stallions refrigerated semen. For this purpose, ejaculated from 15 stallions of the breeds Quarter Horses and Mangalarga Marchador were used. The semen sample was divided into two groups: Group 1 (G1) in which semen commercial diluents skim milk-based semen-Botu ® (Botupharma, Botucatu-SP, Brazil) was added until the concentration of 50 million sperm/mL, and Group 2 (G2) in which spermatic selection, using the density gradient Equipure® with centrifuge force of 300xg, during 20 minutes, and the pellet with the selected sperm was diluted with commercial extender medium skim milk-based Botu-semen®, until the same concentration of G1 was reached. After the seminal processing (H0) and after 24 hours of refrigeration at 15$^{o}$C (H24) were evaluated the spermatic parameters of total mortality (TM, %), progressive motility (PM, %), speed (VAP, μm/s), curvilinear velocity (VCL, μm/s), rapid sperm (RAP, %) by CASA (Computer-Assisted Sperm Analysis) and spermatic membrane integrity (IMP, %) by epifluorescence microscopy. The following parameters presented lower values ($p<0.05$): TM (G1=76.0±9.9$^{a}$ vs G2=83.3±4.8$^{b}$ on H0 and G1=30.0±22.0$^{a}$ vs G2=67.7±19.0$^{b}$ on H24), PM (G1=32.6±8.6$^{a}$ vs G2=43.0±8.5$^{b}$ on H0 e G1=8.1±5.8$^{a}$ vs G2=29.5±13.4$^{b}$ on H24) and RAP (G1=67.9±12.2$^{a}$ vs G2=75.8±6.7$^{b}$ on H0 and G1=23.0±19.3$^{a}$ vs G2=54.6±22.9$^{b}$ on H24) of G1 in relation to G2 at the two moments. The IMP (G1=63.5±16.2 vs G2=70.8±13.2 on H0 and G1=30.0±22.0$^{a}$ vs G2=67.7±19.0$^{b}$ on H24) was higher ($p<0.05$) on G2 when compared to G1. Higher values were also observed for VAP (G1=125.0±14.5$^{a}$ vs G2=134.0±17.8$^{b}$ on H0 and G1=108.4±25.2 vs G2=103.7±23.3 on H24) and VSL (G1=228.8±24.6$^{a}$ vs G2=238.4±27.1$^{b}$ on H0 and G1=210.5±22.0 vs G2=199.5±33.1 on H24) of G2 on H0 when compared to G1. The spermatic selection using Equipure® presented recovery (%) of 41.7±20.1 of the sperm. The results of the present study allow us to conclude that the selected sperm by the Equipure® density gradient presents higher kinetics values and spermatic viability both in the H0 analysis as on the H24. Therefore, this method is an option for possible improvement in the fertility rate of refrigerated semen of stallions.

A010 Male Reproductive Physiology and Semen Technology

# *In vitro* culture of equine spermatogonial cells in a suspension cell system

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**Keywords:** culture, equine, spermatogonia.

The aim of the present study was to develop a method to culture equine spermatogonial cells in a suspension cell system and to induce cell differentiation and spermatogenesis in vitro. For that, testicles obtained from adult horses (between 3 and 4 years) were cut into small pieces and kept under digestion solution (5 mg/mL Collagenase Type I, Gibco™ and 5 mg/mL Hyaluronidase, Sigma™) for 1 hour and 30 minutes at 37oC. After digestion, the samples were passed through a 40 µm filter and then centrifuged to remove the digestion solution and washed in HBSS (Life™). The viability of the cells was checked with Trypan Blue staining. Meanwhile, a 24-well culture plate were prepared with 300 µL of 1.5% agarose gel (Difco Ágar Noble- BD™) in each well forming a thin layer of agar. The obtained cells were diluted in the culture medium which was composed of MEM α (Gibco™), 10% KSR (Gibco™), 50 µg/mL of gentamicine (Sigma-Aldrich™), 3.0 µg/mL of Amphoterecin B (Gibco™), 80µL/mL of ITS (BD™), 50 UI/L of rFSH (Puregon™, Organon), 1 µmol/L of testosterone and Equine Pituitary Extract (EPE). During the first week 3.3 x10-7 M of retinoic acid (Sigma™) and 3.3 x10-7 M of retinol (Sigma™) were added to the medium. Half the volume of the medium contained in each well was changed once a week. The culture system was maintained with 5% CO2 in air at 32$^{o}$C. After 56 days in culture it was possible to see the formation of clusters of spermatogonia and the presence of a lot of cells above the agar layer. Immunofluorescence was used to characterize the presence of spermatid and Sertoli cells. The cultured cells were fixed with 4% paraformaldehyde and permeabilized with saponine (BD Cytofix/CytoPerm) both for 20 minutes and at room temperature. Then, the cells were incubated with the primary antibody for protamine (Protamine 1, M-51, rabbit polyclonal antibody, Santa Cruz Biotechnology™) and for SHBG (M-207, rabbit polyclonal antibody, Santa Cruz Biotechnology™), diluted 1:50, for 18h at 4$^{o}$C. Next step was the incubation with the secondary antibody conjugated with Alexa-Fluor 555 (goat anti-rabbit IgG, Invitrogen™) diluted 1:100, for 1h at room temperature. Nuclear counterstaining was done with DAPI (Invitrogen™). The slides were evaluated under a fluorescent microscope at magnification of 20x and 40x. It was possible to observe a lot of cells fluorescent for protamine showing the formation of spermatid cells under in vitro culture. Negative control was done by incubation with BSA instead of the primary antibody. Positive control was done with the culture of mouse cells under the same conditions. The presence of cells positive for SHBG shows the maintenance of Sertoli cell viability on our culture conditions. Other studies need to be done to check the potencial fertility of these spermatids.

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A011 Male Reproductive Physiology and Semen Technology

# Evaluation of industrialized coconut water and egg yolk citrate as extenders of refrigerated ram semen

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**Keywords:** coconut water, ram semen, termorresistance.

Coconut water industrialized as base for ovine semen extender elaboration, for preservation at low temperatures was tried, searching a seminal extender of easy elaboration and low cost to be used in the farm routine. The experiment was accomplished in the Escola Vila Pepita Farm - UBM - Barra Mansa, RJ, using two rams of the Santa Ines breed, with 24 months of age. The delineation was blocks at random, in factorial arrangement with four treatments (2 extenders x 2 dilution procedures) and 3 preservation periods to 8°C (0, 2 and 24 hours) in 5 blocks (weeks). The ejaculated were obtained by eletroejaculation, being diluted 1:5, in coconut water industrialized extender (50 mL of coconut water industrialized, 25 mL of bidestiled water and 25 mL of sodium citrate solution to 5%) and egg yolk citrate extender (80 mL of sodium citrate solution to 2.94% and 20 mL of egg yolk). The dilution procedures were the semen added to extender versus extender added to semen. The obtained data were submitted the analysis of variance. The of motility (%) and vigor (0-5) characteristics found for the diluted semen and conserved by to 8ºC, up to 24 hours, in coconut water industrialized extender ($52.5^{b}$ and $2.8^{b}$) were inferior ($P<0,05$) to the ones of the egg yolk citrate extender ($76,5^{a}$ and $3.8^{a}$). However up to 4 hours preservation the means were similar (motility of 93.3 and 93.8%, and vigor of 4.8 and 4.9) in coconut water industrialized and egg yolk citrate extenders. Termorresistance tests of diluted semen in the egg yolk citrate showed efficient and superior in supplying energetic and cryoprotective elements for the maintenance of the spermatic viability preservation up to 24 hours at 8ºC, in comparison to diluted in coconut water industrialized. The results obtained for motility and vigor in the diluted and conserved semen up to 24 hours to 8ºC in the extender constituted the coconut water industrialized could have been resulted from alterations in pH and osmolarity of the used solution, once the procedures were recommended for extender elaboration using coconut water *in natura*. Other possible negative influences could have occurred in function of the conservative substance used in the industrialized coconut water. The procedures putting the semen on extender and extender on the semen did not present alterations in motility and in the semen vigor conserved in both extenders.

A012 Male Reproductive Physiology and Semen Technology

# Antioxidants on crioula breed ram semen cryopreservation

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**Keywords:** cryopreservation, ovine, semen.

Antioxidants were used as an additive in ram semen cryopreservation to reduce oxidative stress. This biochemical unbalance is caused by the mitochondria production of reactive oxygen species and free radicals, which can cause damage in cell membrane, affecting parameter like progressive motility. The objective of this experiment was to evaluate the action of different B-mercaptoethanol (BME) concentrations (1mM, 5mM e 10mM) associated or not with 5 mM of cysteine, on Crioula breed ram semen cryopreservation. Rams (n=4) were collected seven times, twice a week, using artificial vagina. Immediately after they were collected, the semen was diluted 1:1 (v/v) with a base extender (tris, egg yolk and glycerol) in isothermal conditions. Using Neubauer chamber, the spermatic concentration was determined to allow equal contribution of each ram in the semen pool. After that, the semen was divided in the following experimental groups: (TC) control, 1mM BME (T1), 5mM BME (T2), 10mM BME (T3), 1mM BME+5mM cysteine (T4), 5mM BME+5mM cysteine (T5), 10mM BME+5mM cysteine (T6). Sperm motility, membrane and acrosome integrity were evaluated before and after cryopreservation. The concentration was adjusted for $100x10^6$ spermatozoa/0.25 mL straw. Data was analyzed by Statistics® (2009) software, no-parametric data with Kruskal-Wallis and parametric data with ANOVA. Membrane integrity was similar between the treatments. After thawing sperm motility presented differences between treatments T2 and T5, with 27.1% and 44.3%, respectively, but no treatments were different from control. For acrosome integrity after thawing, the treatments T2 (33.7%) and T3 (37.4%) were not different from control (33.7%), but the rates shown were higher than T1 (25.4%). These results suggested that in the concentrations used, the antioxidants were not beneficial for the parameters evaluated.

A013 Male Reproductive Physiology and Semen Technology

# Assessment of field fertility and several *in vitro* sperm characteristics of different sires utilized in a timed-AI program

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**Keywords:** bull effect, field fertility, semen analysis.

This study aimed to investigate the conception rate (CR) as well as several in vitro sperm characteristics of different sires used in a fixed-time-AI program. For the field study, all lactating Nelore cows (n=567) were submitted to the same fixed-time-AI protocol. The protocol started (d0) with cows receiving a P4 intravaginal releasing device (Sincrogest®, Ouro Fino Saúde Animal, Cravinhos, Brazil) and 2.0mg of estradiol benzoate (Sincrodiol®, Ouro Fino). The P4 device was removed on d8 and animals received 500µg of d-cloprostenol (Sincrocio®, Ouro Fino), 300UI of eCG (Novormon 5000®, Intervet, São Paulo, Brazil) and 0.5mg of ECP® (Pfizer, São Paulo, Brazil). On d10 cows were inseminated. Frozen semen doses from three Angus bulls and three different batches from each bull were utilized. For laboratory study, semen samples from the same batches utilized in the field experiment were evaluated. The following in vitro sperm characteristics were assessed: computer assisted semen analysis (CASA), plasma and acrosomal membrane integrity by PI/FITC-PSA fluorescent probes association (flow cytometry), lipid peroxidation by fluorescent probe C11-BODIPY581/591 (flow cytometry), sperm morphology (differential interference microscopy), sperm morphometry and chromatin structure (Toluidine Blue staining). Field results were analyzed using the GLIMMIX software (SAS Inst. Inc., Cary, USA) and laboratory results were analyzed by Paired T test using GraphPad Software (GraphPad Inst. Inc., San Diego, USA). According to field results, a bull effect was observed, since bull B presented lower ($P<0.05$) CR (45.50%, n=189) than bulls A (59.17%, n=169) and C (55.55%, n=209). Similarly, some differences were detected in the laboratory analyses. Semen from bull B (which presented lower CR) demonstrated lower ($P<0.05$) total motility (bulls A:51.04%, B:33.59%, C:46.12%), lower ($P<0.05$) progressive motility (bulls A:44.28%, B:27.97%, C:32.02%), reduced ($P>0.05$) percentage of cells presenting intact plasma and acrosomal menbranes (bulls A:39.10%, B:36.47%, C:47.17%), increased ($P>0.05$) percentage of cells presenting lipid peroxidation (bulls A:1.93%, B:5.93%, C:2.50%), increased ($P>0.05$) percentage of cells presenting major defects (bulls A:14.33%, B:23.50%, C:12.17%), higher ($P<0.05$) width/length ratio (bull A:0.518, B:0.532, C:0.522) in morphometric analysis, as well as increased ($P>0.05$) chromatin heterogeneity (bull A:8.14%, B:10.64%, C:8.47%). It was concluded that the sire presenting lower in vivo fertility also presented inferior in vitro semen quality according to the analyses performed. The methodology employed seems to present some relationship with the fertility of bulls used in the AI program. However, further studies that may help elucidate the main causes of differences in bull fertility frequently observed in field trials are required

A014 Male Reproductive Physiology and Semen Technology

# The use of partial least square (pls) for evaluating the importance of *in vitro* sperm characteristics in the prediction of *in vivo* fertility of different sires

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**Keywords:** conception rate, partial least squares, sperm characteristics.

The aim of this study was to assess in vivo fertility and several in vitro sperm characteristics of different sires in order to identify the sperm variables considered important in the prediction of conception rate. Multiparous Nelore cows (n = 97) from a commercial farm were submitted to the same timed-AI protocol. Frozen semen doses from three Angus bulls and three batches of each bull were used. Semen thawing protocol was performed according to the farm routine. The same thawing procedure was repeated in laboratory to simulate the field thawing procedure. The following in vitro sperm analysis were performed: Computer Assisted Semen Analysis, sperm thermal-resistance after 2 hours of incubation, Hiposmotic Swelling Test (HOST), assessment of plasma and acrosomal membranes by PI/FITC-PSA, assessment of sperm plasma membrane stability by Yo-Pro/Merocianin540, assessment of lipid peroxidation by C11-BODIPY581/591, sperm morphology, assessment of sperm morphometry and chromatin structure by Toluidine Blue staining. For statistical analysis, the Partial Least Squares (PLS) was used to explore the importance of the variables in the prediction of the conception rate. The sperm variables were selected according to Wold's Criterion (1994, SAS, 2001) that considers a value lower than 0.8 as "small" for VIP (Variable Importance for Projection). Hence, the following procedure was performed: after a first PLS analysis containing all variables (n = 44), the predictors with VIP < 0.8 were excluded. Then, PLS analysis was performed for a second time (n = 28 variables). Again, the variables with VIP < 0.8 were excluded. Then, PLS was performed for a third time (n = 20 variables) and the predictors with VIP < 0.8 were excluded. After the fourth run of PLS, all remained variables (n = 17) demonstrated VIP ≥ 0.8. Therefore, these were the variables selected as good predictors of field fertility among the several variables evaluated. The following in vitro sperm variables were selected as important predictors of conception rate: Total Motility (TM), Progressive Motility (PM), Beat Cross Frequency (BCF), Rapidly moving cells (RAP), TM_2h, PM_2h, VAP_2h, BCF_2h, RAP_2h, sperm cells with intact plasma membrane after HOST, sperm with intact plasma and acrosomal membranes, sperm with intact plasma membrane suffering lipid peroxidation, total defects, width/length ratio, Fourier 0, Fourier 2 and chromatin heterogeneity. It was concluded that PLS is a suitable statistical method for selecting in vitro sperm characteristics considered important in the prediction of fertility of sires in field conditions.

A015 Male Reproductive Physiology and Semen Technology

# Evaluation of susceptibility of sperm to DNA fragmentation and antioxidant enzymes in different stages of cryopreservation

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**Keywords:** bovine, flow citometry, oxidative stress.

One of the causes of DNA fragmentation is the presence of oxygen reactive species (ROS). The integrity of sperm DNA is fundamental for support embryonic development. Although studies show that bovine spermatozoa has low levels of DNA fragmentation, most of researches are carried out only in pos-thawed sperm. The aims of this study were: 1) evaluate the DNA integrity in 3 moments of sperm cryopreservation process, and 2) check the correlation between DNA fragmentation and antioxidants enzymes. During seven weeks, semen collection from four Holstein Bulls was performed weekly with an artificial vagina. Each semen sample was diluted with commercial extender Botubov® and separated in 3 treatments: 37$^{o}$C semen (fresh), 5$^{o}$C semen (cooled), and frozen at -196$^{o}$C semen (thawed). Each evaluation was made 0h and 2h after incubation in TALP semen medium. In the treatment cooled and thawed, the samples were heated at 37$^{o}$C before evaluation. The test of susceptibility of chromatin to acid denaturation (SCAD) was used to evaluate DNA integrity. The samples were stained with fluorescent probe acridine orange and were analized in the capillary flow cytometer. The activity of antioxidant enzymes catalase, superoxide dismutase and gluthatione peroxidase was made in spectrofotometry (absorbance/minute). Statistic analysis were performed using SAS program (statistical analysis system) evaluating the effects of treatment, time and interaction between treatment x time. When an effect was observed, the test of media comparison was performed. A pos-test was made to check correlation between the variables. There was no difference between treatment and time for antioxidant enzymes. There was difference between treatments ($p<0.05$) and incubation time ($p<0.05$) for SCAD test. Thawed samples (11.21±0.85) had an increase in percentage of positive spermatozoa for this test when compared to the fresh samples (5.13±1.27) and cooled (5.85±1.18). The samples incubated 2h had highest percentage of positive spermatozoa for the test SCDA (9.57±1.14) in comparison to the 0h samples (5.70±0.62). There was no correlation between the variables. These data suggest that the cryopreservation do not interfere in antioxidant citoplasmatic enzymes activity, but induces increased susceptibility to fragmentation of the sperm chromatin.

A016 Male Reproductive Physiology and Semen Technology

# Use of chromomycin A3 staining in ram sperm cells for detection of protamine deficiency by flow citometry

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**Keywords:** protamine, spermatozoa, staining.

The sperm chromatin guarantees the passage of paternal genetic information effectively. Modifications of DNA can cause changes in processes involving reproduction. Changes in DNA integrity may be due to protamine failure. Protamine is a nuclear protein that plays a key role in DNA integrity. The aim of the present study was to investigate protamine deficiency in ram sperm cells using Chromomycin A3 (CMA3) staining by flow citometry. Semen collection from 12 rams, at 7 months of age, was performed for seven weeks. After analysis of progressive motility and sperm concentration, around 30 million sperm cells were washed in Ca and $Mg^{-}$ free phosphate-buffer saline (PBS) and fixed in Carnoy's solution (3:1 methanol: glacial acetic acid) at 4°C. After 10 minutes, the cells were washed again in Ca and $Mg^{-}$ free PBS and distilled water. The pellet was stained with CMA3 solution (0.25 mg/ml in Mcllvaine Buffer). After 20 minutes incubation at room temperature, in the dark, samples were washed again with Ca and $Mg^{-}$ free PBS. After re-suspension in Ca and $Mg^{-}$ free PBS the samples were analyzed in the flow cytometer. In order to check the procedural validity for ram semen samples, a control method was performed, at the same time, using semen smears previously deprotaminated and fixed in Carnoy's, according to Simões et al (2009, Biotechnic and histochemistry 84(3), 79-83). The deprotamination was performed by decondensation of sperm nuclei and protamine extraction, resulting in 37.8 ± 0.70% of protamine deficient cells. The mean of each animal was compared and no statistically significant difference was observed between animals and between weeks analyzed ($p > 0.05$). The mean percentages of all animals obtained in flow cytometry were: 2.15 ± 1.11 for cells stained with CMA3 (with protamine deficiency) and 97.41 ± 1.11 for unstained cells. It was possible to conclude that this technique is very specific, although less sensitive. Due to this fact, it is important to perform this technique with extreme caution. Based on these results, the flow cytometry assessment with CMA3 is a useful tool for detecting sperm protamine deficiency in rams.

A017 Male Reproductive Physiology and Semen Technology

# Serum testosterone concentration in Nelore bulls supplemented with rumen-protected fat

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**Keywords:** diet, metabolism, polyunsaturated fatty acids.

The testosterone metabolism occurs primarily in the liver and their circulating concentrations can be influenced by the amount and type of diet provided. Furthermore, there are reports of the influence of circadian rhythms in the secretion of testosterone in ruminants. Based on evidence that supplementation of rumen protected fat (Megalac-E) may increase the circulating concentrations of steroid hormones (Guardieiro et al., 2010. Pesquisa Agropecuária Brasileira, v.45, p.408-414), this study aimed to evaluate the effect of supplemental fat on serum testosterone concentration in bulls, and to assess whether there is variation in hormone concentrations at different times of day after feeding. Forty-eight Nelore bulls received the same concentrate diets, however, different than Control (C) group (n = 24), bulls in the Fat group (F) were also fed with rumen protected fat rich in linoleic acid (Megalac-E, n = 24, 1.5% in dry matter basis). For circulating testosterone quantification, blood samples were taken before the diet offer, which occurred at 8:00 am (0 h), 4 and 12 h after, in two moments: the adaptation period to the control diet (no fat), and approximately 35 d after the offer of the experimental diets (C vs. F). Serum concentrations of testosterone were measured by radioimmunoassay. For comparison between groups, ANOVA was used. Results are presented as mean ± standard error. During the adaptation period, there was no difference in testosterone concentrations between 0 h (n = 34), 4 h (n = 40) and 12 h (n = 41) in relation to the diet offer (5.2 ± 0.42, 5.1 ± 0.19 vs. 4.9 ± 0.24 ng/mL, respectively, P > 0.10). Similarly, regardless of fat feeding in the second period of evaluation, there was no effect of time on circulating concentrations of testosterone (0 h [n = 47]: 5.0 ± 0.30, 4 h [n = 44]: 4.5 ± 0.28 and 12 h [n = 44]: 4.9 ± 0.22 ng/mL, P > 0.10). Because there was no effect of time, data were grouped to compare circulating testosterone between bulls C vs. G. Contrary to our initial hypothesis, fat supplementation did not increase serum concentrations of testosterone (G [n = 69]: 4.7 ± 0.23 vs. C [n = 66]: 5.0 ± 0.21 ng/mL, P > 0.10). Therefore, this study did not prove the hypotheses that there is effect of diet, time of day or protected fat supplementation on circulating concentrations of testosterone in Nelore bulls.

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A018 Male Reproductive Physiology and Semen Technology

# Comparison between conventional artificial vagina and experimental articulated artificial vagina for equine semen collection

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**Keywords:** artificial vagina, bacteriological exam, semen.

Semen collection made with closed artificial vagina usually exposes the ejaculate at high temperature and high bacterial contamination caused by the contact with latex mucous membrane and the penis rubbing against it. Therefore, semen collection should be performed with adequate equipment so that the semen does not suffer injuries and preserve their fertilizing capacity. The objective of this study was to evaluate two artificial vaginas, a conventional and an experimental articulated, and its effects on semen characteristics in addition to determining the number of bacterial colonies in each vagina. For this purpose, semen from five stallions Mangalarga Marchador, about 5-10 years of age, were evaluated. The ejaculates were collected using a conventional artificial vagina (VAC), Hannover model, to perform closed collection which has only a single container where the semen is deposited. The experimental articulated artificial vagina (VAEA) was used to perform the open collection. The semen volume collected in each vagina was measured and then evaluated the total number of spermatozoa (NTE) using Neubauer chamber. The total motility (MT) and progressive (MP) were analyzed by analysis system (CASA) Hamilton Thorn Research 10.8 Ceros®. For microbiological examination of samples, an aliquot from each ejaculated was diluted NaCl solution (0.9mg/ml) in dilutions of 1:10, 1:100, 1:1000, 1:10.000, 1:100.000, subcultured in Nutrient Agar and incubated at 37$^{o}$C/48h. Data analysis was performed using the Tukey test, considering a 5% significance level comparing the variables. The average volume collected with the VAEA (46.5±12.49 ml) showed statistically higher (P=0.0035) collected with the VAC (34.05 ± 12.65ml). The average of the NTE collected with the VAC (6932 ± 3465.1x106) and VAEA (6590 ± 4146.3x106) showed no significant differences between them (P=0.7785). The average number of jumps was 2.15 with VAEA and 1.95 with VAC, however, did not differ significantly (P=0.5827). The averages MT (71.75 ± 13.01%) and MP (61.50 ± 13.77%) of ejaculates collected with the VAC did not differ significantly (P=0.4319 e P=0.1872) when compared those collected with VAEA (68.25 ± 14.80%) and (55.25 ± 15.60%), respectively. The microbiological results showed uncountable bacterial growth in dilution 1:10 for all stallions collected with VAC maintaining growth through the dilution of 1:10000. With VAEA, growth proved uncountable only two stallions remained in 1:10 dilution to 1:1000 dilution. The experimental articulated artificial vagina was accepted by stallions, because the number of jumps until ejaculation was not statistically different from conventional closed vagina and besides that, it was observed lower bacterial growth, featuring lower health risk for the mare uterus.

A019 Male Reproductive Physiology and Semen Technology

# Influence of high environmental temperatures on reproductive characteristics of Brahman bulls

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**Keywords:** heat tolerance, semen, shadow.

Similar to others tropical countries, in southeast Brazil cattle are bred under environmental conditions. Testicular degeneration promoted by heat is the main cause of infertility in bulls, and reproduction of beef cattle is generally carried out under environmental conditions of grazing system. The aim of this work was to evaluate possible effects of shadow availability on pastures on the reproductive characteristics of Brahman bulls. Ten Brahman bulls aging between 24 and 30 months were used in this study. Before allocating the animals into the experimental groups, three semen samples were collected from each animal for biological evaluation. Five bulls were maintained in a pasture with shadow availability and five bulls were maintained on a pasture without artificial or natural shadow. Relative humidity and temperatures of black globes, under the sun and shadow, and dry bulb were collected during the experimental time by a local weather station. Semen samples were collected every 14 days for 2 months, in a total of 4 semen samples per animal. The semen characteristics evaluated were volume, aspect, mass movement¿, motility, straight movement, sperm concentration and morphological exam. Other reproductive characteristic evaluated was testicular consistence. During the experimental period the environmental temperatures ranged between 34.2ºC and 15.5ºC (maximum and minimum, respectively) with an average of 25ºC, and the minimum Black Globe and Humidity Index (BGHI) calculated was 95.7. Semen turbulence¿, motility and vigor of sperm showed no significance difference (P>0.05) between treatments or sample collection time. Also, no difference was found in testicular consistence regardless of sample collection time. The testicular consistence was measured before the semen collection of each bull. This study simulated the conditions of the bovine natural environment in a grassland-based system. In conclusion, we propose that once no negative effect was observed due to the absence of shadow the evaluated Brahman bulls present good adaptability to the tropical climate. It is suggested, therefore, that further experiments should be conducted in order to evaluate the effect of shadow availability in other taurine breeds exposed to the tropical environment.

A020 Male Reproductive Physiology and Semen Technology

# Effect of nitric oxide enzyme (NOS) inhibition and nitric oxide (NO) elimination on motility patterns and plasma membrane integrity and acrossome reaction of cryopreserved equine sperm

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**Keywords:** equine, L-name, methylene blue

Nitric oxide (NO) is a reactive nitrogen species that may act as an antioxidant or free radical in inter and intra-cellular signaling [Dixit et al, Anim. Reprod. Sci. 2001, 65:1]. The aim of this study was to verify the effect of NOS inhibition and NO elimination on motility patterns, plasma membrane integrity and acrosome reaction in equine cryopreserved sperm. Three ejaculates were obtained from each of three stallions (n=9). Semen was packaged into 0.5-mL straws to a final concentration of 200 x 106 cell/mL in Botu-Crio® extender (Botupharma Animal Biotechnology) and frozen by automated technique using a programmed machine (TK 3000® TK Tecnologia em Congelação – Ltda, Uberaba, Brazil). Four straws, of the same ejaculate were thawed in water bath at 37$^{o}$C/30s, and centrifuged in bovine in vitro fertilization (IVF) media [Gardes et al, Acta Scientiae Veterinariae, 2011, 2011:39(Suppl 1)]. Supernatant was discarded and semen was then incubated in the same media with L-arginine, with or without the NOS inhibitor, Nω-nitro-L-arginina metil ester (L-NAME) and in media with L-arginine with or without the NO scavenger, methylene blue treatments: control T1= (C - IVF), T2= L-arginine 10mM (based in previous experiments), T3= L-NAME 1 mM [Herrero, Free Radic. Biol. Medic. 2000, 29:522-36], T4= methylene blue 100 mM [Donnelly, Mol. Hum. Reprod. 1997, 3:755-62], T5= L-arginine (10 mM) + L-NAME (1mM) and T6= L-arginine (10 mM) + methylene blue (100mM) in 60, 120 and 300 minutes at 38°C under 5 % CO2. Next, computerized analysis of the sperm motility was carried out. To assess the integrity of plasma and acrosomal membranes samples were stained with PSA-FITC and PI probes and analyzed by flow cytometry. Data were analyzed using ANOVA and the means were compared within each time with Tukey test, with a level of significance of 5 %, using SAS software. The removal of NO from the culture medium inhibited sperm cells at all incubation times. Total and progressive motility were reduced in groups T2 and T5= total motility (time 0: T2= 24.89 % ± 2.57 %, T5= 25.78 % ± 2.44 %), progressive motility (time 0: T2= 5.44 % ± 1.39 %, T5= 5.17 % ± 1.14 %). Sperm incubated with the NO scavenger showed a higher percentage of cells with plasma membrane and acrossomal integrity at 60 and 120 minutes of incubation (<0.05). The acrosome reaction was induced when treated with L-arginine (time 300: T2= 7.76 % ± 1.40 %, time 300: T5= 9.42 % ± 1.76 % e time 120: T6= 3.56 % ± 0.51 %). In conclusion, the dose of 10 mM L-NAME was not sufficient to eliminate NOS in equine sperm, and NO elimination maintained the integrity of plasma and acrosomal membrane. Besides these benefits, NO removal totally inhibited sperm motility, suggesting a beneficial role of endogenous NO in preserving the motility of cryopreserved equine spermatozoa.