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Software efficiency for counting sheep spermatid cells: Preliminary results

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This study aimed to develop a software for sheep sperm cell count, from images taken under a microscopy. This program should be compatible with notebooks and PCs for home use, easy to use, in the Portuguese language and free. It is intended, with this software, standardize the sperm concentration analysis carried out by veterinarians during andrological examinations at field, cheapening the cost of acquisition of specific equipment. Ten images of sheep semen were made by microscopy from thawed semen doses. The 0.5 mL reed were thawed for 30 seconds in a water bath at 37°C. Semen drops were analyzed by CASA (Hamilton Thorne Research) and deposited on slides and Neubauer chamber both covered with cover slip for microscopy. The images were obtained from phase contrast microscope (Jenamed2) with 1.3 MP camera attached (Coleman). The software was developed from resources already available in an open source Java solution called ImageJ. The approximate count of the sperm contained in the image was possible through particle analysis capabilities. Initially, the video images were converted into frames and subjected to some treatments, using only 8-bit color and segmenting grayscale so that the software could do the analysis of the image particles. The 10 semen samples were analyzed by CASA, by the technician in Neubauer chamber and by the software for spermatozoa count. For statistical analysis, the results of the counts were subjected to analysis of variance (SAS, 2012) at a significance level of 5%. The average values of the sperm cell counts did not differ and were 186.00 ± 78.63 , 171.00 ± 50.63 and 282.00 ± 155.81 by CASA, by the technician and by the software, respectively ($P > 0.05$). The software was highly efficient for sperm cell count, being a convenient and easy to use solution. The CASA instruments have shown high levels of accuracy and reliability using different methodologies of classification that provide a great tool to improve our knowledge and ability to analyze sperm, making it essential to research, personnel training and standardization between laboratories. Regardless of the manufacturer, the different instruments are based on similar principles, but differ in terms of optics and software used to identify the sperm and the construction of the track, respectively. Our differential is the gratuity and ease of use, since it is a specific software for sperm analysis and available in Portuguese. In conclusion, the developed software showed the same efficiency as the count carried out by CASA e by the technician.



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Evaluation of spermatic characteristics of small and medium-sized dogs: Partial results

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The present study has the objective of evaluate the spermatic parameters of small and medium size dog breeds. Nine adults dogs were used, with good reproductive histories, from private breeders, five (n=5) of medium size Breeds (one American Pit Bull Terrier, one Basset Hound and three Sharpei) and four animals of small Breeds (Poodles), the age was ranging from three to six years, clinically healthy and trained for the semen manual retrieve procedure. The testicles were evaluated in length and width. The semen was obtained by digital manipulation of the genitalia and the parameters volume, color, odor, motility, vigor, concentration and spermatic morphology have undergone descriptive analysis. The mean values±standard deviation (SD) of length and width of the small dogs testicles were 2.17±0.70 and 1.53±0.50 (left testicle) and 2.17±0.47 and 1.50±0.30 (right testicle), respectively, and of the medium sized dogs were 3.64±0.42 and 2.14±0.27 (left testicle) and 3.64±0.54 and 2.08±0.31 (right testicle), respectively, all testicles were considered symmetric after the evaluations. In relation to the spermatic parameters, the mean±SD to the small Breed dogs were volume = 3.75±0.66, color = 50% (2/4) yellowish and 50% (2/4) white, aspect = 100% (4/4) aqueous, odor = 100% (4/4) suis generis, motility = 83.33±5.77%, vigor = 3.33±0.58, spermatic concentration = 380±62.41x10⁶Sptz/mL, major defects = 15.67±5.13% and minor defects = 31.33±4.51%, while the medium-sized dog parameters were volume = 4.20±0.57, color = 60% (3/5) yellowish and 40% (2/5) white, aspect = 80% (4/5) milky and 20% (1/5) aqueous, odor = 100% (4/4) suis generis, motility = 70±4.18%, vigor = 3.50±0.55, spermatic concentration = 439±18.72x10⁶Sptz/mL, only one animal was bellow 200x 10⁶Sptz/mL concentration, major defects = 21.40±5.18% and minor defects = 27.20±13.22%. The morphologic alterations presented in large incidence were strongly folded tail, absence of acrosome, medium piece defects, proximal and distal citoplasmatic droplet, besides the folded tail. The pathology of dog semen limits were not regulated by the MAPA (Brazilian Agricultural Government Ministry) as Bulls and Stallions were, however the Brazilian Animal Reproduction College has the recommendation of total defects maximum in 20%, and the major defects not exceeding 10%. Despite the high incidence of spermatic defects, all animals showed a good history of fertility, with breeding followed by positive pregnancies. It could be concluded that are some variances among the spermatic parameters between small and medium size breeds, some alterations could be explained by the testicle size, which is related to the animal size, therefore more animals should be analyzed in order to obtain an effective comparison among the reproductive parameters of different size dog Breeds.



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Angiotensin-converting enzyme characterization before and after the cryopreservation of Gyr bulls semen

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The aim of this study was to characterize the angiotensin-converting enzyme (ACE) before and after the cryopreservation of Gyr bulls semen. Ejaculates from five sexually mature bulls were used. After semen collection, 1mL aliquot was removed for fresh semen analysis, the rest was submitted to cryopreservation process. Frozen semen sample was maintained in liquid nitrogen until thawing at 37°C for 30 seconds. Both fresh and thawed semen were twice centrifuged with TALP for plasma and diluent withdrawal, respectively. The samples were submitted to western blot, immunocytochemistry and enzymatic activity. The means of the stained areas in immunocytochemistry, mean pixels of the protein bands and the enzymatic activity were submitted to analysis of variance (F test) and the differences between the groups were compared by Student test, considering 5% of significance. After western blot execution with anti-ACE monoclonal antibody it was possible to observe 100 kDa band in the semen extract protein of analyzed bulls. All the observed bands intensity decreased by 70% order ($P<0.05$) after cryopreservation. ACE periacrossomal location was demonstrated by immunocytochemistry and the stained area by fluorescent antibody decreased significantly ($P<0.05$) after cryopreservation. The enzymatic activity evaluated by hydrolysis the furanylacryloyl-L-phenylalanyl-glycylglycine (FAPGG) substrate was significantly lower ($P<0.05$) in cryopreserved semen in relation to the fresh also. It was concluded that cryopreservation process leads to intensity decreases in ACE bands, in the stained area by immunocytochemistry and enzymatic activity of ACE in semen from Gyr bulls.



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Comparison of different surgical procedures to prepare teaser boars

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Besides the production, the swine industry is now facing an important pressure from the consumers to develop better systems to address the animal welfare issues and the use of antibiotic. The use of collective gestational pens by the European Union, have forced producers from different parts of the world, to adapt and to implement to their system. The heat detection procedure also needs some adaptations, since females are moving from individual crates to collective pens during the detection procedure. Therefore, the use of teaser boars could be an alternative to avoid the undesired pregnancy, and facilitating the heat detection procedure in collective pens. Therefore, the objective of this study was to evaluate different surgical procedures to produce boar teasers. For that, 39 male pigs (30-35kg) were used, (9-10 per technique). Animals were kept for 12 hours prior the surgery without food and water. Animals were sedated with tiletamine hydrochloride and zolazepam hydrochloride (Zoletil 50® - 5mg/Kg/IM); azaperone (Strenil® - 2mg/ kg/ IM) and local anesthesia was conducted at the incision line with lidocaine without vasoconstrictor (1,5 mg/Kg). The surgical procedures were: Vasectomized animal via the inguinal access (VI): Removing 2cm of the deferent duct between the last pair of teats. Tail-epididymectomy (CE): removal of the bilateral part of the epididymis tail, using the ventral access of scrotal sac. Vasectomized animal via scrotal access (VE): removal of 2 cm in the funicular portion of the deferent duct using a caudal access of the scrotal sac. To evaluate the surgical procedures, cortisol levels were measured 48hrs after the procedures using radioimmunoassay, and at seven months of age a complete Breeding Soundness Examination test was conducted, including (testicular volume, and testosterone levels measurements). Data were analyzed using PROC MIXED (SAS®) with comparisons using a Tukey test, significance was considered if ($p < 0.05$). During the Breeding Soundness Examination, no changes or reduction of libido was identified due to the surgical procedures. Only one animal from the VE group had spermiation. No differences were observed among the procedures and the control group. The cortisol concentrations were $3.29 \pm 0.47 \mu\text{g/dl}$ (VI); $3.23 \pm 0.47 \mu\text{g/dl}$ (CE); $3.48 \pm 0.27 \mu\text{g/dl}$ (VE) and $3.29 \pm 0.51 \mu\text{g/dl}$ (cont.) ($p=0.98$), testosterone levels $6.25 \pm 1.92 \text{ ng/dl}$ (VI), $5.92 \pm 1.74 \text{ ng/dl}$ (CE); $6.14 \pm 1.82 \text{ ng/dl}$ (VE) and $5.29 \pm 2.02 \text{ ng/dl}$ (cont.) ($p= 0.98$) and testicular volume $343.52 \pm 35.29 \text{ cm}^3$ (VI); $463.05 \pm 33.65 \text{ cm}^3$ (CE); $422.76 \pm 35.29 \text{ cm}^3$ (VE) and $423.70 \pm 37.20 \text{ cm}^3$ (cont.) ($p=0.12$). All the surgical procedures could be used to produce a teaser boar, since they have produced low levels of stress and have not caused any negative effect on the libido and the testosterone levels to the animals.



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Effect of cyclophosphamide associated or not with propolis on the thickness of muscle stroma and height of the prostate secretory epithelium in pubescent guinea pigs

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The prostate in guinea pigs is an odd gland, with flanged edges and presence of a shallow groove (isthmus) on its dorsal surface (Gradela et al. 2013, *Biotemas* 26: 221-31). Prostatic secretions play an important role in semen production (Cepeda et al. 2006, *Int J Morphol* 24:89-97) and ovum fertilization (Carvalho 2005, Tese em Biologia Celular e Estrutural[1], UNICAMP[2]) and the gland is often affected by diseases associated with old age, such as benign prostatic hyperplasia (BPH) (Brianezi et al. 2006, *Braz J Vet Res Anim Sci* 43:65-73; Averbeck et al. 2010, *Rev AMRIGS* 54:471-7) and cancer (Srougi et al. 2008, *Rev Med* 87:166-77). Cyclophosphamide (CF) causes infertility due to overproduction of reactive oxygen species (Abd-Elmoaty et al. 2010, *Fertil Steril* 94:1531-34), while propolis has been recognized as a powerful antioxidant (Russo et al. 2006, *Life Sci* 78:1401-6). However, the effect of CF and propolis on the prostate remains unknown. This study aimed to evaluate the effect of CF alone or associated with ethanolic extract of propolis (EEP) on the thickness of muscle stroma (ST) and height of the secretory epithelium (EH) of prostate in pubescent guinea pigs. This study was approved by Comitê de Ética Experimental em Humanos e Animais[3] of UNIVASF (Protocol nr. 0001/160315). The guinea pigs were divided into six groups: CONT (0.10 ml/10 g of saline PV, N=6); CF100 (100 mg/Kg of CF, N=6); CF200 (200 mg/Kg of CF, N=5); EEP (50 mg/Kg of EEP, N=5); CF100+EEP (100 mg/Kg of CF + 50 mg/Kg of EEP, N=4); CF200+EEP (200 mg/Kg of CF + 50 mg/Kg of EEP, N=5). Treatments were performed once a week/5 weeks. IP administration route was used to saline and CF and gavage to EEP. In the sixth week, animals were anesthetized and euthanized, and the prostate was collected and submitted to routine histological processing with HE staining. ST (μ m) was evaluated with 10x augmentation at four points corresponding to the maximum heights of the wall, not including the mucosa, and EH (μ m) with 40x augmentation in the same areas using an Olympus BH-2 microscope and the Image Pro Plus 2.0 software (Media Cybernetics, Brazil). Data (mean + SEM) were evaluate by ANOVA with test hoc Student's t-test ($\alpha=5\%$) (ASSISTAT 7,6 Beta). Results showed decreased ($P < 0.05$) ST and EH in groups CF100 (514.54 + 38.47 and 202.,51 + 9.,47, ST and EH, respectively), CF200 (563.57 + 33.29 and 217.34 + 3.36); CF100+EEP (490.32 + 24.40 and 192.75 + 12.93) and CF200+EEP (540.85 + 31.80 and 179.06 + 16.94) compared to CONT (660.64 + 34.05 and 252.35 + 22.41). In conclusion, cyclophosphamide alone or associated with propolis decreases the thickness of the stroma and the height of the epithelium in the prostate and it may be beneficial for the treatment of diseases that cause the increase of these parameters such as benign prostatic hyperplasia and prostate cancer.

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[1] Cell and Structural Biology Thesis; [2] Universidade Estadual de Campinas – University of Campinas; [3] Committee on Experimental Ethics in Humans and Animals; [4] Conselho Nacional de Desenvolvimento Científico e Tecnológico - Brazilian Council of Scientific and Technological Development.



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Effect of cyclophosphamide associated or not with propolis on the projection of the folds of the tunica mucosa and height of epithelial cells of vesicular gland in pubescent guinea pigs

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Guinea pigs have even, tubular, craniocaudally elongated vesicular glands, with smooth surface and consistent appearance located at the origin of the pelvic urethra, being the largest accessory glands (Gradela et al. 2013, *Pesq Vet Bras* 33:942-8), whose secretions are essential for reproduction (Wong et al. 2001, *Reprod Toxicol* 15:131-6). Cyclophosphamide (CF) causes gonadal failure with oligozoospermia or azoospermia and even irreversible infertility (Freire et al. 2006, *Rev Bras Reumatol* 46:12-20), however, its effect on the vesicular glands has not been studied, as well as the effect of natural antioxidants such as propolis, which improved the function and integrity of sperm DNA (Safarinejad; Safarinejad 2009, *J Urol* 181:741-51). This study evaluated the effect of CF alone or associated with ethanolic extract of propolis (EEP) on the projection of the folds of the tunica mucosa (FP) and height of epithelial cells (EH) of vesicular gland in pubescent guinea pigs. This study was approved by Comitê de Ética Experimental em Humanos e Animais[1] of UNIVASF (Protocol nr. 0001/160315). Guinea pigs were divided into six groups: CONT (0.10 ml/10 g of saline PV, N=6); CF100 (100 mg/Kg of CF, N=6); CF200 (200 mg/Kg of CF, N=5); EEP (50 mg/Kg of EEP, N=5); CF100+EEP (100 mg/Kg of CF + 50 mg/Kg of EEP, N=4); CF200+EEP (200 mg/Kg of CF + 50 mg/Kg of EEP, N=5). Treatments were performed once a week/5 weeks. IP administration route was used to saline and CF and gavage to EEP. In the sixth week, animals were anesthetized and euthanized, and the prostate collected and submitted to routine histological processing with HE staining. FP (μ m) was evaluated at six fields per animal using 10x augmentation, while EH was determined in 40x augmentation at the same points using an Olympus BH-2 microscope and the Image Pro Plus 2.0 software (Media Cybernetics, Brazil). Data (mean + SEM) were evaluated by ANOVA with test hoc Student's t-test ($\alpha=5\%$) (ASSISTAT 7,6 Beta). Results showed marked decrease ($P < 0.05$) of FP in animals treated with CF200+EEP (954.05 + 153.11) compared to CONT (1628.83 + 211.58) and EH in animals treated with CF200 (149.81 + 4.59); CF100+EEP (128.64 + 18.70) and CF200+EEP (108.90 + 14.25) compared to CONT (212.00 + 13.08). In conclusion, cyclophosphamide decreases the projection of the folds of the tunica mucosa and height of the secretory epithelium at the 200 mg dose and height of the epithelium in both doses when associated with propolis, confirming its toxic effect on vesicular cells.

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[1] Committee on Experimental Ethics in Humans and Animals; [2] Conselho Nacional de Desenvolvimento Científico e Tecnológico - Brazilian Council of Scientific and Technological Development.



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Effect of diet containing gossypol on testicular histology in Santa Inês rams

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The gossypol is a Polyphenolic pigment present in all parts of the cotton seed plant and its derivatives. Earlier experiments showed side effects in caused by feed containing high percentage of cottonseed and claim that gossypol might affect reproductive system, causing abnormalities in sperm and subsequent infertility. The objective of this work was to evaluate the effects caused by a diet rich in cottonseed gossypol on testicular histology and seminal parameters of hair sheep gossypol Santa Inês Breed. For that, 22 rams, same age, which were divided into 2 groups, being the animals confined in individual pens. The gossypol Group (Gg = 15), supplemented with 500 g of cottonseed and the control group (Gc = 7), supplemented with 500 g of corn for 104 days and subjected to semen evaluation before (7 days), during (at intervals of 15 days) and after (10 days) the period of supplementation. The cottonseed used was analyzed to the levels of free gossypol by CBO laboratory (Campinas – SP), founding 3,39 g/Kg of gossypol of cottonseed. No statistical difference was observed in sperm parameters between Gg and Gc during all the experiment. After the end of the supplementation period, 5 animals (2 from Gc and 3 from Gg group) were submitted to unilateral orchiectomy. The material was used for conducting comparative Histopathological examination in historesin, No difference was seen in the diameter of seminiferous tubule, between control and gossypol group, however it showed a statistical difference in height of the seminiferous epithelium, having a larger epithelium in the Gg compared with control group. The height of the seminiferous epithelium is an effective feature for assessment of sperm production in mammals, however in this study there was no significant difference in sperm parameters between control and treatment group. Our results showed that supplementation with cottonseed did not influence sperm quality, while the testicular morphology despite having demonstrated a significant difference in height of the seminiferous epithelium, did not affect sperm quality in vitro. Further studies may be conducted by increasing the duration of treatment to see whether there may be a cumulative effect.



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Effect of the selenium supplementation on the ovine spermatic DNA integrity

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The present study aimed to evaluate the effects of different concentrations of selenium (Se), at Ovine nutritional supplementation, on its Spermatozoa DNA integrity. Thirty male ovine were used, aging from 18 to 24 months, housed in an intensive system of creation, divided in five experimental groups, control group (CG; n=6), with supplementation at mineral salt without Se added, G1 (n=6), same mineral salt mixed with 5mg of Se (Sodium selenite)/Kg, G2 (n=6), control mineral salt mixed with 10mg of Se/Kg, G3 (n=6), control mineral salt mixed with 15mg of Se/Kg, and, G4 (n=6), control mineral salt mixed with 20mg of Se/Kg. In every group was respected an adaptation period of 14 days, following a treatment time of 56 days. The samples were obtained by electroejaculation, before starting treatment and after the end of the treatment, totaling 30 ejaculates per experimental group. The DNA fragmentation detection of the semen samples were performed using the Comet Assay, adapted for ram semen (Martins et al. Ani. Reprod., v.10, 697-703, 2013). The experimental were implemented using a Latin Square 5x5, i.e., five treatments and five experimental times. The dates were evaluated using GLM process (SAS, 2009). The mean differences were compared using Tukey's test at 5% of significance. The mean percentage of DNA integrity of CG were 12.34±2.21%a, G1 = 6.60±0.59%b, G2 = 6.38±0.71%b, G3 = 6.74±0.64%b, and, G4 = 6.52±0.51%b (P < 0.05). The CG had shown a higher percentage of DNA fragmentation when compared to the other groups, but no statistical difference were observed among the Se supplemented groups. According the results presented in this study, the Selenium supplementation (at the presented experimental conditions) had shown a benefic effect on the DNA integrity preservation at ovine spermatozoa. Financial support: FAPESP (no. 2011/51503-7) and CAPES (post-Doctoral support).



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Effect of different doses of cyclophosphamide associated or not with propolis on the tubular diameter and height of cells of the epididymal duct in pubescent guinea pigs

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Sperm maturation occurs in the body of the epididymis (Herms; Robaire 2002, *The epididymis: from molecules to clinical practice*, p.81-102), thus, changes in its function cause infertility (Jarvi 2012, *Fertil Steril* 97:1298). Cancer in young adults increases every year and the success of treatments has made studies on the impact on future fertility relevant (Anders; Souza 2009, *Cienc Cuid Saude* 8(1):131-7). Cyclophosphamide (CF) causes oligozoospermia and even irreversible infertility (Freire et al. 2006, *Rev Bras Reumatol* 46:12-20), however, its effect on the epididymis is still unknown, as propolis, which has a protective effect on human sperm (Russo et al. 2006, *Life Sci* 78:1401-6). This study evaluated the effect of CF alone or associated with ethanolic extract of propolis (EEP) on the tubular diameter and height of the cells of the initial segment (distal portion of head and body) and tail of the epididymis in pubescent guinea pigs. This study was approved by Comitê de Ética Experimental em Humanos e Animais[1] of UNIVASF (Protocol nr. 0001/160315). The animals were divided into six groups: CONT (0.10 ml/10 g of saline PV, N=6); CF100 (100 mg/Kg of CF, N=6); CF200 (200 mg/Kg of CF, N=5); EEP (50 mg/Kg of EEP, N=5); CF100+EEP (100 mg/Kg of CF + 50 mg/Kg of EEP, N=4); CF200+EEP (200 mg/Kg of CF + 50 mg/Kg of EEP, N=5). Treatments were performed once a week/5 weeks, using IP administration route to saline and CF and gavage to EEP. In the sixth week, guinea pigs were anesthetized and euthanized, and the right epididymis was collected and submitted to histological processing with HE staining. The tubular diameter of the epididymal duct (TD, μm) was evaluated on 10x augmentation and the height of the epithelium (EH, μm) on 40x augmentation using an Olympus BH-2 microscope and the Image Pro Plus 2.0 software (Media Cybernetics, Brazil). A total of 24 measurements per segment, 72 measurements per animal and 216 measurements per group were performed. Data (mean + SEM) were evaluated by ANOVA with test hoc Student's t-test ($\alpha=5\%$) (ASSISTAT 7.6 Beta). Results showed decreased ($P < 0.05$) TD in the initial segment of the epididymis in groups CF200 (1085.48 + 84.51), CF100+EEP (919.65 + 131.31) and CF200+EEP (969.50 + 72.33) and in the tail only in CF200 (1244.94 + 69.79) compared to CONT (1491.64 + 119.81 and 1619.15 + 129.16, initial and tail, respectively). There was EH decrease ($P < 0.05$) in the initial segment in groups CF100 (480.46 + 16.68), CF200 (618.54 + 74.07), EEP (675.45 + 62.47), CF100+EEP (554.68 + 92.24) and CF200+EEP (467.73 + 4.00) and in the tail in EEP (486.79 + 26.91) and CF200+EEP (233.59 + 24.52) compared to CONT (913.31 + 83.01 and 651.80 + 57.10, respectively). In conclusion, cyclophosphamide was toxic specially for the initial segment of the epididymis, and propolis, in the dose tested, had no protective effect against this action, increasing this effect in some cases.

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[1] Committee on Experimental Ethics in Humans and Animals; [2] Conselho Nacional de Desenvolvimento Científico e Tecnológico - Brazilian Council of Scientific and Technological Development.



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Heat shock effect on redox balance and acrosomal reaction in Holstein bulls spermatozoa

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Heat stress affects spermatogenesis and compromises sperm quality. However, the direct effect of elevated temperature on spermatozoa is not fully understood. The natural or artificial insemination of animals under heat stress condition, exposes the spermatozoa to temperatures above of physiological in the female reproductive tract, which can compromise the spermatozoa quality and fertilization capacity. Therefore, the present work objective was determine the effect of heat shock (in vitro heat stress) on the acrosomal reaction and the production of reactive oxygen species (ROS) in bovine spermatozoa. It has already been shown that protein kinase C (PKC), involved in the regulation of acrosomal reaction, is also activated by oxidative stress. Thus, PKC role was evaluated in this process. Straws from five Holstein bulls (pool of 2 bulls/replica) were thawed at 37°C and subjected to Percoll gradient. The pellet was diluted in SP-TALP at 2.5x10⁶sptz/mL. After dilution, spermatozoa were incubated for 4 hours according to the following treatments: control 0hr (non-incubated), 35°C (testicular temperature control), 38.5°C (body temperature control) and 41°C (heat shock). The acrosomal membrane integrity (FITC-PSA 100 µg/mL for 10 min) and ROS (CellROX® Green 5 µM for 30 min) were analyzed by flow cytometry (BD FACSCALIBUR) and the data obtained were analyzed by the FlowJoLLC program V.10. Phosphorylated PKC was assessed by immunofluorescence using the rabbit antibody anti-phosphoPKC (1:100) and the secondary antibody Alexa Fluor-555 anti-rabbit (1:200). DNA stained with Hoechst 33342 (5 µg/mL). The PKC (15 µM bisindolylmaleimideII: BIMII) inhibitor was used to determine the PKC role on spermatozoa acrosomal reaction subjected to heat shock. Data were analyzed by ANOVA (parametric data) and Wilcoxon test (non-parametric data) using SAS JMP Statistical Discovery 11.0. The incubation of spermatozoa at 35°C (67.9 + 12.3 %), 38°C (81.7 + 12.3 %) and 41°C (86.9 + 12.3 %) increased (p<0.05) ROS production when compared to control group 0h (8.9 + 12.3 %). However, the magnitude of this increase on ROS production was higher (p<0.01) in spermatozoa incubated at 41°C when compared to 38°C. Similarly, incubation of spermatozoa at 38°C (30.5 + 1.45 %) and 41°C (51 + 1.45 %) induced (p<0.001) acrosomal reaction when compared to 35°C (22.2 + 1.45 %) and 0hr (7.4 + 1.45 %). Inhibition of PKC with BIMII did not affect acrosomal reaction at any temperature evaluated, suggesting that PKC is not involved in this process. To confirm this result, the phosphorylated form of PKC was evaluated. Immunofluorescence results showed that sperm incubation at 35°C (24232,838 + 926,10883), 38.5°C (21479,378 + 926,10883) and 41°C (21452,572 + 926,10883) increased (p<0.01) phosphorylation of PKC when compared to group 0h (18249,092 + 926,10883). However, phosphorylation of PKC did not differ between spermatozoa incubated at 38.5°C and 41°C. In conclusion, the increase in temperature results in a higher amount of ROS and acrosomal reaction by a pathway independent of PKC.



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Effect of bovine oviductal fluid obtained in the follicular or luteal phase of the estrous cycle on the ram sperm function and capacitation

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Despite advances in the use of *in vitro* capacitating substances in other species, in small ruminants the presence of estrous sheep serum is essential to achieve ideal IVF rates. In attempt to mimic the sperm capacitation process that occurs *in vivo*, this study aimed to evaluate the effect of oviductal fluid on ram sperm function and *in vitro* capacitation. Oviducts from cows were collected in regional slaughterhouses and classified as follicular or luteal phase. Then the oviductal fluid obtained was stored at -20 °C until its use. Subsequently, semen was collected from three rams simultaneously forming a pool. After sperm selection (swim up), sperm were incubated in different media, using Fert-TALP (positive control; commonly medium used for IVF) as the base medium (1) or with the following changes: Fert-TALP (2) without capacitating substance (negative control), (3) without capacitating substance and supplemented with 10% of oviductal fluid in the follicular phase and (4) without capacitating substance and supplemented with 10% of oviductal fluid in the luteal phase. The sperm were incubated at 38 °C in 5% CO₂ and the parameters of sperm kinetics, plasma membrane (PM) integrity and capacitation status were evaluated after 0, 2, 4, 6, 18 and 24 h. The variables were subjected to ANOVA and Tukey analysis ($P < 0.05$). There was no difference ($P > 0.05$) among all treatments during incubation for PM integrity. At 2 h and 4 h of incubation, the negative control showed lower ($P < 0.05$) values in the velocimetric parameters (VSL, VAP, LIN, STR, WOB, ALH e BCF) and progressive motility. Media supplemented with oviductal fluid (both phases) were greater in these parameters, compared to the negative control. At 6 h, there was no difference ($P > 0.05$) among all treatments. At 4 h and 24 h, the negative control showed higher ($P < 0.05$) capacitation rate compared to other groups. At 18 h, the positive control showed higher ($P < 0.05$) values in the velocimetric parameters (VCL, VSL, VAP, LIN, STR, WOB, ALH e BCF) compared to other groups. At 18 h and 24 h, sperm incubated with oviductal fluid in the follicular phase presented higher ($P < 0.05$) acrosome-reacted cells compared to other groups. In conclusion, supplementation with 10% of oviductal fluid regardless the phase of estrous cycle to the IVF medium, promotes an improvement in the velocimetric parameters and sperm kinetics for up to 4 h of incubation. This strategy can be considered as a possible alternative because it presents lower cost compared to defined and synthetic additives and, due the simplicity of the technique in relation to the extraction and purification process, necessary to obtain estrous sheep serum.

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Effects of nutritional supplementation with polyunsaturated fatty acids on seminal quality and fecundity of Nelore males classified for residual feed intake

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Nutrition has great influence on sperm production and quality. Thus, diets with polyunsaturated fatty acids (PUFAs) may exert a positive effect on the seminal quality of bulls. In addition to diet, food efficiency is another important factor for genetic breeding programs, and the indicator "residual feed intake" (RFI) may have effects on the reproductive performance of bulls. Within this context, the objective of the present study was to evaluate the effects of long-term dietary supplementation with PUFAs (200g/animal/day Megalac®) in Nelore bulls classified for RFI on the quality of frozen semen and fertility (evaluated by *in vitro* production of embryos - IVP). Twenty-four young bulls from the Animal Science Institute (IZ) of Sertãozinho, SP, were used. The animals remained in performance test from 7 months to 12 months of age for determination of RFI, being classified as low RFI (< average - 0.5 standard deviation of the mean (SD)) and high RFI (> average + 0.5 SD). The animals were then divided into 4 treatments (n=6 bulls per treatment): 12 low RFI animals receiving control diet or supplemented with AGPs, and 12 high RFI animals receiving control diet or supplemented with AGPs. Experimental diets were isoproteic and were formulated to meet the nutritional requirements of young bulls growing on pasture (*Brachiaria brizantha* cv. Marandu pasture in continuous stocking). The animals were 14.3 ± 0.13 months old at the beginning of the experiment and initial weight was 389.5 ± 5.43 kg. The semen of all 24 animals was frozen at the end of work (1 single ejaculate of each bull) when they reached 24.6 ± 0.13 months of age. Samples of thawed semen were submitted to computerized analysis of sperm kinetics (CASA, Hamilton Thorne Research, IVOS-14, USA) and IVP. The parameters evaluated were: total motility (TM%), progressive motility (PM%), rapid cells (RAP%), lateral displacement of sperm head (ALH, μm), beat frequency (BCF, Hz), cleavage rates (%) and rates of embryonic development to the blastocyst stage (%). The data were submitted to analysis of variance by SAS proc MIXED with significance of $P < 0.05$. No differences were observed between the treatments for the post-thawed semen analysis by CASA, and the means between the 4 groups were $75.5\% \pm 6.5$ MT, $57.2\% \pm 5.8$ MP, $71.5\% \pm 6.6$ RAP, 5.8 ± 0.2 μm ALH and 26.2 ± 1.47 Hz. There were also no differences between treatments for cleavage rates and blastocyst rates, and the means between the 4 groups were $78.2\% \pm 2.1$ (3922 cleaved oocytes, out of a total of 5014 matured in 12 replicates) and $35.8\% \pm 2.1$ (1799 embryos produced out of a total of 5014 matured oocytes), respectively. It is concluded that the supplementation with sources of AGPs for bulls classified as high and low RFI did not influence the post-thawing sperm characteristics and the fecundity of the semen evaluated by the *in vitro* production of embryos. Thus, further studies are needed to determine the real influence of AGPs and RFI on sperm quality and on IVP.

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***In vitro* effects of bovine semen cooling and cryopreservation**

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The FTAI protocols, when well implemented, result on high percentages of ovulations (70 to 90%), occurring in a window of approximately 48 hours. However, the pregnancy results show a large amplitude (30 to 65%), part of these results may also be in response of the semen quality used. With the hypothesis that refrigeration causes less damage to sperm than cryopreservation, an *in vitro* experiment was carried out to compare the effects of refrigeration and cryopreservation of bovine semen. Eighteen ejaculates of 10 Nelore bulls were used. After the semen collection, each ejaculate was fractionated in two equal aliquots on 50 mL conical tubes, pre-diluted in Botubov® medium (BB, Botupharma/Brazil). One sample was subjected to refrigeration (5°C) for 48 hours and the other was cryopreserved. Both semen samples were diluted (40x10⁶ sperm/straw), filled in 0.5 mL French straws (IMV® Technologies/France) and placed into Botuflex® (Botupharma/Brazil) refrigeration boxes for evaluations at the 24, 36 and 48 hours of refrigeration. Another 10 straws were submitted to the cryopreservation process on an automated freezing system (TK 3000®/Brazil). Post-refrigeration and cryopreservation semen samples were evaluated in duplicate by computerized system of sperm movement (SCA program - Sperm Class Analyzer, Spain), sperm abnormalities by differential interference contrast chamber (DIC), membrane integrity evaluation and acrossomal, mitochondrial membrane potential by epifluorescence microscopy with the fluorescence probes Hoescht 33342, Propidium iodide, FITC-PSA and JC-1. The fixed effects of the cooling times were evaluated, besides the effects of the cryopreservation vs refrigeration evaluated, using PROC GLIMMIX of the SAS. After 24 hours of refrigeration at 5°C, the semen presented a decrease on progressive motility (from 69.59 ± 3% pre-refrigeration to 50.36 ± 5%) and on percentage of spermatozoa with intact plasma and acrossomal membranes and high mitochondrial potential (PIAIA from 55.7 ± 5% to 33.7 ± 5%). Any other alterations were found up to 48 hours at 5°C. When comparing the sperm characteristics of cooled semen for 48 hours at 5°C (REF) with cryopreserved semen (CRIO), it was observed that the refrigeration presented higher values only for VCL (CRIO = 106.04 ± 6 and REF = 152.5 ± 5 μm/s); (CRIO = 69.3 ± 4 and REF = 50.4 ± 1%), WOB (CRIO = 79.4 ± 4.8 and REF = 60.6 ± 1%), and cryopreserved semen showed higher values for BCF (CRIO = 23.6 ± 0.48 and REF = 13.46 ± 4.42). However, the values of total motility, progressive motility, percentage of fast cells, VSL, VAP and percentage of cells with plasma membranes integrity, acrossomal and high mitochondrial potential were similar between refrigerated and cryopreserved samples.



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Influence of seasonality in the physiological parameters and buffalo seminars in the West Region of Pará

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The presente study was undertaken with the objective of evaluating the influence of climatic variables on parameters of ejaculates of Murrah buffalo bulls raised in humid tropical Amazonian climate, evaluate some physiological parameters such as heart frequency (HF), respiratory frequency (RF), rectal temperature (RT) and superficial temperature (ST) during the rainy and non-rainy periods in Amazon region. Five buffaloes (n=5), average of age 2.5 ± 0.5 years and body weight 450.0 ± 35.5 kg were selected from a group of 12 adult bulls. In order to characterize the study area, a mini-station was installed at the experiment site to measure ambient temperature, relative humidity, rainfall, Solar radiation and wind speed. The experiment was carried out in the municipality of Santarém, Meso-region of Lower Amazonas, West of Pará State, with experimental animals kept in lairage regime. The animals had constant access to fresh and clean water, as well as mineral salt ad libitum in a trough. The sanitary control was carried out according to pre-established criteria for the species. The experimental period was divided into two phases: rainy season (RS), from February to May, and non rainy season (nRS), from August to November 2016. The evaluations physical and morphological of the semen samples were performed with fresh semen, immediately after each collection. The immediate analyzes pertinent to the physical and morphological characteristics of the ejaculates were carried out and corresponded in the RS volume of 3.4 ± 2.0 mL; mass activity of 4.4 ± 0.5; motility of 80.4 ± 5.6%; vigour of 4.4 ± 0.4; concentration of 657.300 ± 237.865,1 x10⁶sptz / mL; major defects of 9.0 ± 2.6%; minor defects of 11.2 ± 3.9%; total defects 2of 0.2 ± 5, 3% and sperm plasma membrane integrity (SPMI) 84.8 ± 5.6%, where as in the nRS, the results were 4.0 ± 2.1 mL; mass activity of 3.0 ± 1.0; motility of 56.2 ± 13.4%; vigour of 3.0 ± 1.0; concentration of 586,000 ± 291,925.9 x10⁶sptz / mL; major defects of 20.8 ± 9.9%; minor defects of 27.5 ± 6.3%; total defects 48.3 ± 9.3% and SPMI of 57.9 ± 12.4%. Furthermore, it was observed a statistical difference (P<0,01) for the parameters mass activity, motility, vigour, major defects, minor defects, total defects and sperm plasma membrane integrity between the both periods. The data on heart frequency, superficial temperature (head, back, groin and scrotal pouch) showed statistical difference between both periods (P<0,01). However, the relative data, for RF and TR did not show any difference between both periods (P>0,01). The HF data were 54 ± 10.9 bpm and 48 ± 11.8 bpm; RF 22 ± 6.8 mpm and 23 ± 6.7; RT, 38.3 ± 0.8 °C and 38.2 ± 0.8 °C; ST, in the head region (STH) were 33.3 ± 2.5 °C and 36.2 ± 2.4 °C; in the back region (STB) 33.6 ± 2.1 °C and 35.1 ± 2.1 °C; in the groin (STG) 32.3 ± 2.2 °C and 34.6 ± 2.2 °C; and scrotal pouch (STS) 30.3 ± 2.0 °C and 32.3 ± 2.0 °C, RS and nRS, respectively. The physiological parameters that were influenced by the non-rainy period, compared to the rainy season were heart rate and surface temperature, of head, back, groin and scrotal pouch (p<0,01).



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Embryo production using epididymal sperm submitted to different selection methods and their influence on the embryo sex

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Epididymal spermatozoa and their use in assisted reproductive technologies (RT), such as IVP, have an important role in the multiplication of genetic material from sires that die suddenly and/or have acquired reproductive failure. However, in order to establish an appropriate procedure to use those sperm in embryos IVP, a better knowledge about their physiological behavior facing events involved IVP, such as different methods of sperm selection, is needed. The aims of this study were evaluated different methods of sperm selection for IVP procedures and their influence on the embryo sex. A pool of epididymal (EP) and ejaculated (EJ) cryopreserved spermatozoa, recovered from seven Gir bulls through electroejaculation followed by bilateral orchiectomy were used. The pool of the two groups were selected by three different methods: Percoll gradient 45%90% (GE Healthcare Bio Science, Uppsala, Sweden), PureSperm gradient 40%80% (Nidacon Laboratories AB, Gothenborg, Sweden) and wash in Tyrode's Albumin Lactate and Pyruvate (SpTALP). Four groups were formed: ejaculated on Percoll (EJ-P), control group; epididymal on Percoll (EP-P); epididymal on PureSperm (EP-PS) and epididymal on SpTALP (EP-T). After selection, sperm samples were co-incubated with a total of 759 cumulus-oocyte-complexes (COC's) in fertilization medium in 7 replicates experiment. Embryos were evaluated two days (D2), six days (D6), seven days (D7) and eight days (D8) after fertilization and then, embryos were storage for sex evaluation. Embryo sexing was performed according to Sousa et al (Theriogenology, 90, p.25, 2017), by PCR technique. Embryo rates data were analyzed using Chi-square (mean±SD; P<0.05) and sexing date by Wilcoxon using Prophet 5.0 (mean±SD; P<0.05). Cleavage rates (D2) and blastocyst rates on D6 were higher for EP-PS group (80% and 48%, respectively) than the other groups. At the D7 and D8, blastocyst rates were similar (P>0.05) between EP-P (D7 54%; D8 55%) and EP-PS (D7 37%; D8 37%) groups. EP-T and EJ-P groups showed similar blastocyst rate in D6 (27%; 32%), D7 (37%; 44%) and D8 (37%; 45%), which was lower than the others groups that used EP sperm. Male and female embryos showed differences only in EP-P group (38% and 62%, respectively). For others methods of sperm selection differences were not significant (P>0.05). These results suggest that PureSperm and Percoll were the better methods of sperm selection, for embryos production using EP. Furthermore, the relation between male × female embryos only showed differences when Percoll gradient was used, resulting in more female embryos. Financial support: CNPq and Embrapa.



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How much sperm can we collect from the epididymides of Crioulo stallion??

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Stallion sperm spermatozoa capable of fertilization can be harvested from the cauda epididymis and be used for artificial insemination (Barker & Gandier, 1957) of a limited number of mares or for cryopreservation, being the last chance to obtain viable doses from a valuable stallion. The expansion of Crioulo breed is growing fast over the years and still little research is done regarding reproductive parameters, especially with epididymal stallion sperm. The aim of this experiment was to evaluate number of total spermatozoa collected during harvesting Crioulo stallions epididymides. In order to perform the evaluation, 15 Crioulo stallions, aging 4-18 years, were submitted to elective bilateral orchietomy. The epididymis (n=30) were washed with ringer lactate and the cauda of epididymis was isolated. The connective tissue was carefully dissected and the cauda was straightened, as long as possible. The sperm harvesting was performed by a retrograde flushing technique and the smaller parts of the cauda by a flotation technique, with skim-milk based extender. Sperm concentration was measured by computer-assisted semen analyses (CASA - AndroVision®, Minitub, Tiefenbach, Germany) and total sperm count was calculated from volume (mL) of spermatozoa with extender x sperm concentration (per mL). Descriptive statistical analyses was performed with the program Statistix 9® (Statistix. Statistix 9 for Windows. Analytical Software, Tallahassee, FL, USA, 2008). Similar with what happens with ejaculates, the total sperm count presented a large variability among stallions. Minimal sperm count was 1.225×10^6 ; maximal was 12.500×10^6 and medium was 4.122×10^6 spermatozoa per epididymis. Minimal sperm count was 2.450×10^6 ; maximal was 22.500×10^6 and medium was 8.245×10^6 spermatozoa per pair epididymis of the same stallion. The epididymides evaluated from the same stallion had similar size, even so, it was observed a variation of up to 45% in the total sperm count, when compared spermatozoa collected from each epididymides. According to Amann et al. (1979), the two cauda epididymides of a normal, sexually rested, adult stallion can contain 54×10^9 sperm or approximately 61% of the sperm in the excurrent duct system. Bruemmer (2006) reported that 5 to 25 breeding doses, consisting of 800×10^6 sperm per dose, are typically obtained from a given stallion, resulting in $4.000-20.000 \times 10^6$ spermatozoa harvested from the two epididymides and $2.000-10.000 \times 10^6$ per epididymides. In the present study with only Crioulo breed horses, large variations in the total sperm recovery were also reported. According to the literature and the present findings, it is possible to affirm that the variability between individuals is very evident. If you considered the variation of total sperm count between 4.000 to 20.000×10^6 (Bruemmer, 2006) it is observed up to 5x times difference, if considered the variation between 2.450×10^6 and 22.500×10^6 it is observed almost 10x times the difference. In conclusion, the number of spermatozoa that can be collected from adult Crioulo stallions had a huge individual variation, from minimal 2.450×10^6 up to maximal 22.500×10^6 spermatozoa per stallion, including both epididymides.



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Sperm subpopulations defined by sperm head morphometry and the relationship with fertility *in vivo*

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The presence of subpopulations of a sample semen, defined by the sperm head morphometry to reduce the *in vitro* fertility. (Santos, et al. 2016. VI ISABR), But no studies have evaluated this effect on fertility *in vivo*. The objective of the present study was to identify and compare sperm subpopulations (SBP) distributed according to the sperm morphometry in the semen of bulls with high and low fertility. Seven batches of 3 bulls Aberdeen Angus of high fertility (FA) determined by the fertility score (0,536; 0,495 e 0,284) and pregnancy rate (PR: $54.32 \pm 1.60\%$) and 6 batches of low fertility bulls 3 (FB) with negative fertility score (-1.579, -1.758 and -2.447) with lower PR ($41.25 \pm 3.47\%$). Data from 29,939 IATF for the group of FA and 3,259 IATF for the bulls of BF. The semen batches were thawed ($37^\circ\text{C} / 30\text{ sec}$) and a sample was diluted and fixed in formal saline 4% (37°C), one drop ($2.5\ \mu\text{l}$) being placed between slide and cover slip. Images of at least 200 spermatozooids were obtained from each batches and analyzed individually 2,841 spermatozoa using a phase contrast microscope (1000x, Eclipse E200, Nikon®) and a digital video camera (Eurekam 5,0). Sperm head morphometry was automatically analyzed using Image J. software. All parameters have been activated in the measurement set. Data were analyzed by multivariate statistics using the SAS FATSCLUS tool, using the k-means method. PROC MIXED was used to make inferences of each parameter evaluated, considering the effects of fertility, SBP and fertility interaction x SBP, in addition to the random effects of animal and residue. In case of significant effects, the Tukey Test (5%) was adopted. Four SBPs were defined by the area (pixels²), perimeter (pixels), height (pixels) and circumference (pixels) of the spermatid head. The area was the main variable that explained the variance of SBP ($R^2 = 0.83$). SBP1, 2, 3 and 4 varied in the head area in both groups ($P < 0.05$). The spermatid head area for SBP1, SBP2 and SBP3 was similar among the AF groups (SBP1 = 27092 ± 606.87 , SBP2 = 26199 ± 611.72 , SBP3 = 29719 ± 606.08 pixels²) and BF (SBP1 = $27373 \pm$ SBP2 = 27410 ± 606.07 , SBP3 = 29629 ± 623.22 pixels², however, SBP4 was lower ($P < 0.05$) for the AF group (21564 ± 728.93 pixels²) than for the BF (27000 ± 616.71 pixels²). It was also noticed a higher frequency ($P < 0.05$) of SBP4 for BF semen (6.5%) than for AF (3.32%). SBP4 is characterized by being the population of cells that have the smallest area and the largest perimeter, height and circularity of the other SBP ($P = 0.05$). Despite the smaller area for AF, the algorithm that forms the SBPs included other parameters of the morphometry to characterize the SBP4 that should be considered. The data suggest that there are changes in sperm DNA that lead to changes in sperm head morphometry. It is concluded that the higher SBP4 frequency influences on fertility.



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Prolonged use of supplementation with fatty acids in Nelore bulls raised in pasture system improves the frozen semen?

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The production and quality of semen are influenced by several factors, especially nutrition. Thus, diets with polyunsaturated fatty acids (PUFA) have an effect on the reproductive performance of males. Within the context, the objective was to study the effects of prolonged use of supplementation with PUFA (200g/animal/day Megalac®) on the quality of frozen semen of Nelore bulls in pasture. Twenty-four young bulls from the traditional herd of the Animal Science Institute (IZ) of Sertãozinho, SP, were used. The animals were divided into 2 groups: with (n=12) and without supplementation with PUFA (n=12). The animals were kept in *Brachiaria brizantha* cv. Marandu pasture in continuous stocking during the experimental period. The animals were 14.3 ± 0.13 months of age at the start of the experiment and reached 24.6 ± 0.13 months of age at the end and initial weight of 389.5 ± 5.43 kg. Animals from the group supplemented with PUFA received 1 kg/animal/day, the control group received supplementation without PUFA of 1.25 kg/animal/day. Supplements were initially formulated to meet the nutritional requirements of growing young bulls (NRC, 2000; level 2), according to the nutrient content of the pasture, and for them to be isoprotic. For this, the animals in the control treatment received 0.25 kg supplement / animal / day more than the animals treated with PUFA. Samples of thawed semen were submitted to computerized analysis of sperm kinetics (CASA, Hamilton Thorne Research, IVOS-14, USA). The material was previously homogenized and evaluated in a Makler chamber preheated at 38°C, five random fields were observed for each sample and a minimum number of 150 cells per field. The parameters evaluated were: total motility (TM%), progressive motility (PM%), rapid cells (RAP%), path velocity (VAP, $\mu\text{m/s}$), straight velocity (VSL, $\mu\text{m/s}$), curvilinear velocity (VCL, $\mu\text{m/s}$), lateral displacement of sperm head (ALH, μm), beat frequency (BCF, Hz), straightness (STR%) and linearity (LIN%). The data were submitted to analysis of variance by SAS proc MIXED with significance of $P < 0.05$. The post-thawed semen evaluation, the sperm velocity analyzed by CASA was higher for bulls supplemented with PUFA (82.0 ± 1.8 , 68.6 ± 1.2 and 132.0 ± 3.9 for VAP, VSL, and VCL, respectively) in relation for bulls without AGPs (76.2 ± 1.8 , 64.2 ± 1.2 and 122.3 ± 3.9 , respectively) ($P < 0.05$). Despite these differences in sperm velocity, evaluations are needed in conjunction with other seminal tests, especially in natural service and artificial insemination breeding programs, to determine the actual influence of PUFA on sperm quality.

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Resistance Doppler index of stallions treated with human chorionic gonadotrofin (hCG)

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The use of hCG may stimulate the steroidogenic response of Leydig cells and it is used in insufficient testosterone production and cryptorchidism (LIMA et al., Brazilian Journal of Vet and Animal Science, v.37, p.52-55, 2000). Analysing these factors, this research aimed to evaluate possible changes in the resistance index (RI) of the testicular artery and in parameters of the reproductive behavior in the semen collect from fertile stallions after hCG administration. The study was developed during two cycles using four Mangalarga Marchador stallions at the four seasons (January, April, July and October 2016), in Rio de Janeiro, Brazil. The animals were separated in two groups: GI (n = 4), administering 5ml of saline and GII (n = 4), administering 5000 IU of hCG (5ml) (Chorulon® - MSD Animal Health, Brazil), endovenous (ev), in bollus. In each month, the procedures were subdivided into two cycles (C1 and C2) during six days (D) each and with a three days off between cycles. On odd days two stallions were evaluated (one from each group) and on even days the others two remaining stallions from each group. For C2, the crossover experimental rotational scheme was applied, where: CI = animal 1 (GI) and 2 (GII) evaluated on days D1, D3 and D5 and animal 3 (GI) and 4 (GII) on D2, D4 and D6; CII = animal 1 (GII) and 2 (GI) evaluated on days D1, D3, D5 and animal 3 (GII) and 4 (GI) on D2, D4 and D6. The treatment was conducted only on the first day of each cycle (D1 and D2). Following the treatments and with the animals contained and relaxed using Xylazine Chloridrate 2% (0.00028 mg/kg), ultrasonography (US) was performed in spectral Doppler, measuring the RI in the testicular artery one hour before and immediately after semen collect. During the semen collect, Flehmen's reflex frequency and number of mating without erection, reaction time to erection and duration of mating were evaluated. All data were submitted to chi-square and ANOVA tests with significance of 5%. No statistical differences ($P > 0.05$) was observed for the following variables: testicular artery RI, Flehmen reflex frequency, number of mating without erection, reaction time to erection, duration of mating between hCG or saline group, as well among the four seasons. We concluded that administration of a single dose of 5000 IU of hCG in stallions was not enough to change the blood flow in testicular artery, measured by the resistance index, and also did not cause behavior changed in the experimental conditions studied.