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The in vitro impact of the herbicide Roundup® on human sperm motility and sperm mitochondrial functionality

G Anifrandis[1], G.S. Amiridis[2], L. Gavriil[3], V. Gorgogetas[3], K. Dafopoulos[1], A. Daponte[1], E. Dovolou*[2], E. Kachpani[1], Z. Mamouris[3], C.I. Messini[1], A.M. Psarra[3]

[1]Department of Obstetrics and Gynecology, ART Unit, University of Thessaly, School of Health Sciences, Faculty of Medicine, Larissa, Greece; [2]Department Obstetrics and Reproduction, Veterinary Faculty, School of Health Sciences, Karditsa, Greece; [3]Department of Biochemistry and Biotechnology, University of Thessaly, School of Health Sciences, Larissa, Greece.

Keywords: Roundup, sperm motility, mitochondrial functionality.

Common toxicants, such as herbicides, are considered as potential disruptors of sperm parameters and fertility. The aim of the present study was to investigate the impact of a minimum concentration of the widely used herbicide Roundup on sperm motility of semen samples derived from men that were seeking semen analysis prior to IVF. It was also investigated whether this effect was correlated to mitochondrial dysfunction of spermatozoa. A total of 66 semen samples were retrospectively investigated after a written informed consent was taken. Fresh semen samples were collected after 48 to 96 hours of abstinence and were allowed to liquefy at 37°C for 15 to 20 minutes. Each sample was divided into two equal portion; the first portion served as untreated control and in the second 1ppm of Roundup (corresponding to 360ng/ml of the active ingredient –glyphosate) was added. After 1 and 3 hours of incubation, semen analysis was performed in terms of volume and concentration in combination with assessment of the percentage of progressive motile (PRM), non-progressive motile (NPM) and immotile (IM) spermatozoa. Mitochondrial functionality was assessed by fluorescence microscopy analysis using specific mitochondrial dye (CMX). Demographic data, sperm characteristics (volume, concentration and motility) were normally distributed (one sample Kolmogorov-Smirnov test) and statistical analysis was performed by paired t-test. Roundup had profound and deleterious effect on sperm progressive motility (PRM) the first hour of incubation [Control (1h): 46.42% ± 16.2 vs Roundup (1h): 35.26 ± 15.2%, $P < 0.05$], in comparison with the effect after 3h of incubation [Control (3h): 36.86 ± 13.4% vs Roundup (3h): 30.53 ± 11.6%; Mean difference 1h: 11.16% vs mean difference 3h: 6.33%, $P < 0.05$]. The relative fluorescence intensity per unit area (RFU) of mitochondria in the mid-piece region of Roundup-treated spermatozoa was significantly reduced after the first hour of exposure compared to relative controls (0.66 + 0.49 vs 1.21 + 0.95, $P < 0.05$). At the same time, sperm nuclear integrity was not evidently affected. We infer that direct exposure of low Roundup concentrations to semen samples has diverse effects on sperm motility that is correlated to mitochondrial dysfunction.

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Evaluation of the sperm maturation in swine and mouse by flow cytometry using aniline blue as fluorochrome

A. Chacón de Castro*, I. Ortega-Molina, E. Gómez-Fidalgo, R. Sánchez-Sánchez, A. Gutiérrez-Adán, S. Pérez-Cerezales

Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria; Departamento de Reproducción Animal, Madrid, Spain.

Keywords: Aniline blue, sperm maturarion, flow cytometry.

In recent years, the importance of using sperm samples with high chromatin integrity has been shown to be critical to ensure the production of high quality embryos by artificial reproductive technology. For decades, aniline blue staining has been used for the evaluation of sperm maturity. Under bright field microscopy immature spermatozoa are stained in blue by the affinity of aniline blue to the histones. Since the evaluation of the stained sample is biased by subjectivity of the observer and staining intensity is variable between preparations, aniline stains are abandoned nowadays in favour to more objective methods to evaluate sperm chromatin integrity and DNA fragmentation. Here we show an unknown property of aniline blue staining where mammalian spermatozoa exhibit red (>590 nm) fluorescence when excited with green light (510–560 nm). Accordingly, we propose to use this fluorescence for a fast and objective evaluation of sperm maturity in mouse and swine as well as potentially in other mammals.
Mouse sperm was extracted by squeezing from caput and cauda epididymis from 5 B6D2 mice. Swine sperm samples were daily obtained by the gloved-hand technique (4 animals) for 8 days. Aniline staining was performed for all the sperm samples following standard procedure and was analysed by bright field and fluorescent microscopy using a Nikon optiphot-2 microscope (Nikon, Tokyo, Japan). Fluorescence was also analysed by flow cytometry (Beckman Coulter, CA, USA). The DNA fragmentation of caput and cauda mouse spermatozoa was analysed by neutral comet assay following standard procedure.
We found that in both mouse and swine spermatozoa the intensity of aniline blue fluorescence was opposite to the intensity of the blue coloration exhibited under bright field observation. We found that mouse spermatozoa extracted from caput epididymis exhibited less fluorescence than spermatozoa extracted from cauda epididymis (40 ± 4% *vs* 58 ± 5% of stained spermatozoa respectively (n = 5); P = 0.015 according to two-tailed Student's *t*-test). Furthermore, spermatozoa from caput epididymis showed significantly more DNA fragmentation than spermatozoa from the cauda epididymis (8.1 ± 0.7 *vs* 5.5 ± 0.3 % of fragmented DNA evaluated by comet assay respectively (n = 4); P = 0.04 according to two-tailed Student's *t*-test). In addition, we found that by the daily sperm extraction during 8 days from the same swine, the percentage of immature spermatozoa increased in latest ejaculates reporting less aniline blue fluorescence (51 ± 3% at day 7 *vs* 33 ± 7% at day 8 of stained spermatozoa (n = 4); P = 0.03 according to two-tailed Student's *t*-test). In conclusion, we have validate a new method for a fast and reliable evaluation of the level of sperm chromatin maturation by using aniline blue as a fluorochrome and by its analyses by flow cytometry.

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Effect of adding Rosa canina extract and Ascorbic Acid as natural and Synthetic antioxidants on freeze-thawing process of ram semen

H. Daghigh Kia*, S. Razavian, H. Vaseghi Dodran, A. Najafi

Department of Animal Science, University of Tabriz, Tabriz, East Azarbayejan, Iran.

Keywords: Ram Sperm, cryopreservation, oxidative stress.

The extra production of free radicals during the freeze-thaw process is one of the reasons for reducing sperm fertility. Reactive oxygen species (ROS) with penetration to sperm cytoplasm and damaging its membrane can cause sperm death. Rosa Canina herb contains high levels of phenolic compounds such as Quercetin, ellagic acid and Kaempferol. Ascorbic acid plays an important role in scavenging of ROS. The aim of this study was to investigate the antioxidant effect of different levels of Rosa Canina (0, 100, 150, and 200 µl/mL) and Ascorbic acid (0. 0.5, 1, 1.5, 2 mg/ml) on spermatozoa in tris-yolk based diluents. Ejaculates were collected twice a week from the rams using an artificial vagina, during the breeding season (autumn to early winter) and the semen pooled to minimize individual variation. Only ejaculates containing spermatozoa with volume: ≥0.75 ml; motility: ≥80%; abnormal morphology ≤10% and sperm concentration: $\geq 3\times10^{9}$ ml^{-1} were used. Immediately after collection, the ejaculates were immersed in a warm water bath at 34°C until their assessment in the laboratory. A Tris-based extender was used as the base extender. Experimental treatments included different levels (0, 5, 10, and 15 µl/ml) of Rosa Canina extract and Ascorbic acid. Diluted semen was aspirated into in 0.25-mL French straws (4×10^{8} mL^{-1}, spermatozoa per straw), sealed and equilibrated at 4°C for 2 h. After equilibration, the straws were frozen in liquid nitrogen vapor for 7 min in liquid nitrogen and plunged into liquid nitrogen for storage. The frozen straws were thawed individually at 37°C for 30 sec in a water bath for semen evaluation. The assessment of motility parameters was carried out using CASA. The viability, membrane integrity of sperms and lipid peroxidation were evaluated using eosine-nigrosin staining, hypo osmotic swelling test and measuring of malondialdehyde (MDA) concentration, respectively. Each treatment was replicated 5 times. Data were analyzed by SAS (9.1.3) software using the GLM procedure. The results showed that, the percentage of total motility in 150 µl/ml of Rosa canina extract and 1.5 of ascorbic acid was significantly higher compared to the control group (57.4 and 60.2 and 44.6 respectively). The percentage of viability in 150 µl/ml of Rosa canina extract and 1.5 of ascorbic acid was significantly higher compared to the control group (61.9 and 64.2 and 49.1 respectively). The percentage of plasma membrane integrity in 150 µl/ml of Rosa canina extract and 1.5 of ascorbic acid was significantly higher compared to the control group (52 and 54.7 vs. 39.6 respectively). The concentration of MDA in 100 µl/ml of Rosa canina extract and 1.5 of ascorbic acid was significantly higher compared to the control group (18.5 and 19.8 vs. 23.5 respectively) ($P < 0.05$). Our results confirm effectiveness of Rosa Canina extract and Ascorbic acid on microscopic parameters of freezing- thawing ram sperm.

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Could an extra long-term boar semen extender be successfully used during liquid storage of ram semen at 15°C?

B. El Amiri*[1], A. Ben Moula[1], A. Badi[1], K. El Khalil[1], L. Allai[1], E. Gergatz[2], E. Gyökér[2], O. Szenci[3]

[1]INRA, Centre Régional de la Recherche Agronomique de Settat, Settat, BP= 589, Morocco; [2]Biotechnical Research Station, Pharmagene-Farm Ltd., Mosonmagyaróvár, Hungary; [3]SZIU Large Animal Clinical Research Group„ Üllo, Hungary.

Keywords: Duragen, Skim milk, Tris egg yolk.

The aim of the present study, was to verify, whether, a commercial diluent (Duragen: an extra long-term boar semen extender) could be a good alternative to skim milk (SM) and Tris-egg yolk (TEY) to store ram semen at 15°C. Ejaculates were collected once a week during 6 weeks from two different local Moroccan breeds (Boujaad a non-prolific and INRA180 a prolific sheep) using an artificial vagina. The ejaculates containing spermatozoa with >80 % total motility and concentrations higher than 3 x 10^9 spermatozoa/ml were pooled. The pool was divided to three parts and each part was extended in TEY, SM or Duragen (Duragen; Magapor S.L.; Zaragoza, Spain) to reach a final concentration of 0.8 × 10^9 spermatozoa/ml. The Sperm motility (proportion of total and progressive motile sperm) was assessed by means of a computer-assisted semen analysis (CASA) (ISAS, version 1.0.17, Proiser, Valencia, Spain) at different periods (0, 8, 24h). The sperm viability (VIA), the morphology of abnormal spermatozoa (ABN); the hypoosmotic swelling test (HOST); the spontaneous lipid peroxidation (thiobarbituric acid reactive substance: TBARS) and DNA fragmentation tests are in progress to complete the evaluation of semen quality. All data were analyzed using the Statistical Analysis System software JMP (SAS version 10) and expressed as the mean ± SEM. Differences with values of $P < 0.05$ were considered to be statistically significant. The results of progressive motility (PM) showed that at 0h, there was no different between SM (66.12 ± 0.89%) and Duragen (66.52 ± 0.85%) while the TEY showed the lowest value (54.06 ± 1.71%). At 8h of storage, the best PM was recorded with SM (63.57 ± 0.93%), while Duragen showed 58.90 ± 1.19% and TEY 35.69 ± 1.63%. After 24h of storage, the same tendency was recorded. The SM showed 59.53 ± 1.63% of PM while Duragen gave 48.89 ± 1.51% and TEY showed 34.06 ± 2.05%. Similarly, even at 24h of storage, the SM and TEY gave the best results on terms of total motility (TM) (78.48 ± 1.48%, 76.97 ± 1.44% respectively) while Duragen showed the lowest value (68.41 ± 1.47%). In conclusion, Duragen could be a good alternative to TEY and SM regarding the PM. However, it will not be a good one regarding the TM for both extenders (SM and TEY). Additional tests such as VIA, ABN, HOST, TBARS and DNA fragmentation are in progress to complete the evaluation of semen quality.

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Effect of adding Cornus mas extract as a natural antioxidant and BHT on freezing/thawing process of ram semen

D.K. Hossein*, Z.G. Fatemeh, N. Abozar, H. Vaseghi Dodran

Department of Animal Science, University of Tabriz, Tabriz, East Azarbayejan, Iran.

Keywords: sperm, free radicals, *Cornus mas.*

During the freeze-thaw process sperm quality, viability and fertility are reduced because of physiological and chemical stresses on the sperm cell membrane. *Cornus mas* herb contains high levels of phenolic compounds such as Gallic acid, and polyphenols such as Rosmarinic acid and Quercetin. Butylated hydroxytoluene (BHT) is a synthetic analogue of vitamin E that inhibits the auto-oxidation reaction by converting peroxy radicals to hydroperoxides. BHT has been tested successfully to preserve liquid semen and minimize cold shock damage. The purpose of this study was to investigate the effect of *Cornus mas* extract as a natural antioxidant and BHT on frozen-thawed semen quality of ram semen. In this study, five ram were used for semen collection twice a week using an artificial vagina, during the breeding season (August to October) and the semen pooled to minimize individual variation. Only ejaculates containing spermatozoa with volume: ≥0.75 ml; motility: ≥80%; abnormal morphology≤10% and sperm concentration: $\geq 3\times10^{9}$ ml^{-1} were used. Different levels of extract of *Cornus mas* (100, 150 and 200 µl/ml) and BHT (1, 1.5, 2 and 3 mM) were added to Tris-yolk based diluents. Following cooling and freezing of semen samples, they were stored in liquid nitrogen until evaluation. After freezing-thawing, the dynamic parameters were evaluated using CASA system, the viability of sperms using eosin-nigrosin stain, membrane integrity using hypo osmotic swelling test and lipid peroxidation by measuring of malondialdehyde concentration. Each treatment was replicated 5 times. Data were analyzed by SAS (9.1.3) software using the GLM procedure. The results showed that, the percentage of total motility in 150, 200 µl/ml *Cornus mas* extracts was significantly higher compared to the control group (59.2, 52.4 and 44.6 respectively). The percentage of viability in 150, 200 µl/ml *Cornus mas* extracts was significantly higher compared to the control group (63.1, 56.6 and 50.5 respectively). The percentage of plasma membrane integrity in 150, 200 µl/ml *Cornus mas* extracts was significantly higher compared to the control group (54.8, 48.4 and 40.4 respectively) ($P < 0.05$). The percentage of total motility in 1.5, 2mM BHT was significantly higher compared to the control group (56.2, 65.7 and 44.6 respectively). The percentage of viability in 1.5, 2mM BHT was significantly higher compared to the control group (60.5, 69.9 and 50.3 respectively). The percentage of plasma membrane integrity in 1.5, 2 mM BHT was significantly higher compared to the control group (52.5, 61.5 and 40.3 respectively) ($P < 0.05$). Our results confirm the effectiveness of Cornus mas extract and BHT on microscopic parameters of freezing-thawing ram sperm.

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Morphological and functional characteristics of the epididymal sperm derived from the European bison (*Bison bonasus*) of the Altaic population

B.S. Iolchiev, G.N. Singina*, V.A. Bagirov, E.N. Shedova, A.B. Lopukhov, P.M. Klenovitskiy, S.S. Danch, M.A. Zhilinskiy

L.K. Ernst Institute of Animal Husbandry, Podolsk, Russia.

Keywords: epididymal sperm, European bison, in vitro test.

The aim of the present research was to study the morphology, motility, viability and fertility characteristics of epididymal sperm of the European bison. The epididymes were obtained following a forced slaughter (as a result of severe injury) of two bulls from the Altaic population aged 5-7 years. The sperm was collected by scraping the inner surface of the epididymes and assessed for motility and concentration. All procedures were performed within 1 h. Thereafter, the sperm was diluted with the lactose-yolk-glycerol medium to $40x10^6$ spermatozoa/ml and equilibrated for 4 h at 4°C. Sperm aliquots (0.2 ml) were frozen in liquid nitrogen vapor for 5 min and then plunged into liquid nitrogen for storage. Prior to analysis, frozen semen was thawed in pre-warmed medium for 1 min at 37°C. The post-thawed sperm morphometry was performed with help of NIS Elements BR software (Nicon, Amsterdam, Netherlands), the motility was evaluated using computer-assisted semen analysis (Videotest, St. Petersburg, Russia). The sperm viability was assessed with Sperm VitalStain (Nidocon, Mölndal, Sweden) and the fertilizing capacity was determined using a heterologous IVF system. Each analysis was conducted by a single investigator. For *in vitro* test, slaughterhouse-derived cattle oocytes were subjected to IVM procedure described previously (Singina et al., Reprod Fert Dev, 26:154, 2014). The sperm was prepared by the swim-up method. Matured oocytes (n = 234, 35-40 oocytes per group) were co-incubated for 18 h with sperm (1×10^6 spermatozoa/ml) in 500 µl of TALP containing 10 µg mL^{-1} heparin, 20 µM penicillamine, 10 µM hypotaurine, and 1 µM epinephrine at 38.5°C and 5% CO_2 in humidified air. At the end of co-incubation, a part of oocytes was examined for the penetration rate (the number of oocytes having enlarged sperm head(s) or male pronucleus(ei)) by cytological analysis. At Days 2 and 7 after insemination, the sperm fertilizing capacity was determined using morphological and cytological evaluation of cleavage stages and blastocysts formation. Data expressed as means±SEM were processed by SigmaStat software. The morphometric assay (750 spermatozoa per bull, magnification 400x) demonstrated that the average length, head length, head width, head perimeter, head area and tail length of the European bison epididymal spermatozoa were 68.7 ± 12.5 µm, 8.5 ± 0.7 µm, 4.7 ± 0.4 µm, 24.0 ± 1.6 µm, 35.4 ± 5.7 $µm^2$, and 57.3 ± 9.0 µm, respectively. The post-thawed motility reached 40.0 ± 3.3 % (n = 3), whereas the viability was 34.4 ± 4.1 % (n = 3). After heterologous fertilization of bovine oocytes, the penetration, cleavage and blastocyst rates were high and reached 93.3 ± 1.7, 76.6 ± 1.9, and 27.9 ± 2.7 % (n = 5), respectively. The findings of the present research demonstrate that the post-mortem collected epididymal sperm of European bison may be used for creating a bank of genetic resources when breeding this endangered animal species.

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Moxifloxacin effects on ram frozen-thawed sperm function

F. Kountouris[1], A. Gómez[1], J. Gadea*[1,2]

[1]Faculty of Veterinary Science. Excellence Campus for Higher Education and Research "Campus Mare Nostrum". University of Murcia, Spain; [2]Institute for Biomedical Research of Murcia (IMIB-Arrixaca), Spain.

Keywords: antibiotics, bacterial contamination, spermatozoa.

The control of bacteria present in ram seminal samples is important in order to avoid deleterious effect on sperm functionality during storage and transmission of venereal diseases. Previous studies have shown the presence of Mycoplasma agalactie (Ma) in goat and ram seminal samples and its ability to survive in diluted semen (de la Fe et al. Theriogenology 72: 1278, 2009; Gómez-Martín et al. Theriogenology 83: 911, 2015; Prats-van der Ham et al. Theriogenology 2016 doi: 10.1016/j.theriogenology.2016.02.033). Conventional antibiotics used in semen extenders are not effective against Ma or have a detrimental effect on sperm cells. Preliminary results from our lab show that moxifloxacin has antibacterial effect on mycoplasma when used at 0.3 µg/mL (Martínez-Fresneda, unpublished data). However, there is no information about the use of moxifloxacin in semen extenders and its likely effect on sperm cells. The aim of this study was to analyze the effect of moxifloxacin on ram sperm functionality measured in terms of motility, viability and acrosome status. Commercial frozen semen straws from 4 Assaf rams were thawed at 37ºC and incubated for 2 h in Sperm-Talp media supplemented with 0 µg/mL (MOXI-0), 0.3 µg/mL (MOXI-3) and 0.9 µg/mL (MOXI-9) of moxifloxacin. All groups had gentamicine (50 µg/mL) according to Sperm-Talp formulation. Motility and motion parameters were measured by a CASA system, and simultaneously viability and acrosome status by flow cytometry after propidium iodide and PSA-FITC staining (6 replicates). Data were analysed by two-way ANOVA (treatment and incubation time) and when ANOVA revealed a significant effect ($P < 0.05$), values were compared by Tukey test. Results showed that after incubation the percentage of live spermatozoa with altered acrosome, including those with spontaneous acrosome reaction, was higher in MOXI-9 group than in MOXI-0 group (2.7 ± 0.2 vs. 1.8 ± 0.1, $P < 0.01$). Besides a different pattern movement was observed with an increase in the sperm velocities (µm/s) in MOXI-9 group compared to MOXI-0 group (curvilinear-VCL 131.3 ± 2.7 vs. 117.0 ± 3.1; Straight line-VSL 91.8 ± 2.7 vs. 76.7 ± 2.2; Average path-VAP 105.7 ± 2.9 vs. 87.9 ± 2.7, $P < 0.01$). No differences were found neither for viable sperm with intact acrosome (mean value 46.3±2.0%), nor for percentage of motility (mean value 29.2 ± 1.1) nor for other motion parameters. A further study of sperm subpopulations was done by using cluster analysis (Abaigar et al. Biol. Reprod. 60: 32, 1999) grouping spermatozoa in three clusters: slow, medium and fast spermatozoa. Results showed a higher proportion of fast spermatozoa in MOXI-9 group than in MOXI-0 (48.8 vs. 24.3%), and a lower proportion of medium (27.9 vs. 43.9%) and slow spermatozoa (23.2 vs. 31.7%). It can be concluded that under our experimental conditions, moxifloxacin has no effect on sperm functionality when used at low concentration (0.3 µg/mL), but it modifies the pattern of sperm motility and spontaneous acrosome reaction at higher ones (0.9 µg/mL).

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Phosphatidylserine translocation during sperm capacitation is modulated by eNOS in porcine

R. López-Úbeda, R. Diego, C. Matás*

Physiology of Reproduction Group. Veterinary School, University of Murcia, Campus Mare Nostrum, Murcia, Spain.

Keywords: nitric oxide, sperm capacitation, phosphatidylserine translocation.

Reactive oxygen species (ROS) play a key role in the process of sperm capacitation and/or apoptosis [Aitken, J Androl; 13:36-42, 2011]. Nitric oxide (NO) synthesis is mediated by nitric oxide synthase (NOS), responsible for the conversion of L-arginine to L-citrulline [Funahashi, Reproduction; 124: 857-864, 2002]. NOS exists in three isoforms (eNOS, iNOS and nNOS) that have been identified in porcine oocyte and sperm, but many of its functions remain unknown.

The aim of this study was to analyse the effect of NO on the externalization of phosphatidylserine (PS), a process that occurs during sperm capacitation due to destabilization of the membrane [Flesch, Journal of cell science; 114: 3543-3555, 2001].

The role of NO was studied using a NO donor, S-nitroso-glutathione (GSNO; 50µM) or by inhibiting its synthesis employing two different NOS inhibitors: 10mM L-NAME (eNOS inhibitor) and 10mM aminoguanidine (AG) (eNOS and iNOS inhibitor). Sperm cells washed by discontinuous gradient of Percoll, were incubated with the different treatments for 1 hour. Washed sperm but untreated were employed as a control group. Translocation of PS residues to the outer leaflet of the plasma membrane was detected by Annexin V-Cy3TM Apoptosis Detection Kit (Sigma, Madrid, Spain). For this assay, 1µl Annexin V in 450 µl of binding buffer (commercial kit) was mixed with 50 µl of each sperm sample. After 10 minutes of incubation in the dark, at room temperature, samples were fixed with 10 µl formaldehyde (10% in PBS). Sperm with PS exposed (Annexin +) were visualized in red fluorescence (rhodamine filter) [Marti, Anim Reprod Sci; 106: 113-32, 2008]. The data were analyzed by ANOVA ($P < 0.05$).

Our results showed that CONTROL (37.7%), GSNO (38.0%) and AG (36.3%) groups were not statistically significant for staining with Annexin V ($P < 0.05$). However, the sperm incubated with NOS inhibitor (L-NAME) had a lower translocation of PS (29.8%, $P < 0.05$).

These data reveal the possible involvement of NO in the PS translocation during sperm capacitation and that eNOS isoform plays a modulation role.

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Post-thaw changes in sperm membrane and ROS following cryopreservation of dairy bull semen using four different commercial extenders

S. Miguel-Jiménez*[1], T. Mogas[1], A.I. Peña[2], C. Tamargo[3], C.O. Hidalgo[3], R. Muiño[2], J.E. Rodríguez-Gil[1], R. Morató[1]

[1]Departamento de Medicina y Cirugía Animales, Universidad Autónoma de Barcelona, Cerdanyola del Valles, Barcelona, Spain; [2]Departamento de Patología Animal, Universidad de Santiago de Compostela, Santiago de Compostela, Lugo, Spain; [3]Área de Selección y Reproducción Animal, Servicio Regional de Investigación y Desarrollo Agroalimentario, Gijon, Spain.

Keywords: sperm viability, acrosome integrity, flow cytometry.

Semen diluents containing egg yolk as a cryoprotectant may pose hygienic risks and are difficult to standardize. Therefore as an alternative to replace the component of animal origin, egg yolk-free extenders have been developed. This study was designed to compare the effect of three yolk-free extenders (Andromed®, Bioxcell® and Optixcell®) and an egg yolk based diluent (Triladyl®) on post-thaw quality of bull spermatozoa. A total of five ejaculates were collected from six healthy Holstein Frisian bulls (2 to 4 years old) of proven fertility with the aid of an artificial vagina. Each ejaculate was divided into four aliquots and diluted at room temperature with one of the four cryopreservation extenders, allow to equilibrate at 4°C for 4 h and packaged in 0.25 ml straws. Finally, semen was cryopreserved in liquid nitrogen following a standard procedure applied to a computerized freezing processor. For semen analysis, 30 straws from each treatment were thawed for 40 sec at 37.5°C in a water bath as previously described (Muiño et al. Anim Reprod Sci. 109:27-39. 2008). Flow cytometry analyzed parameters were plasma membrane integrity, through the SYBR-14 and propidium iodide (PI) stain, acrosome membrane status by fluorescein isothiocyanate-peanut agglutinin (PNA) and PI stain, $\cdot O_2$ intracellular levels through the hydroethidine (HE) and YoPro-1 stain and H_2O_2 levels by the 2,7-dichlorodihydrofluorescein diacetate and PI stain. Differences among groups were performed through an ANOVA, followed by the Tukey's post-hoc test. Significance was set at two-tailed $P < 0.05$. Semen samples frozen with Optixcell® triggered significantly higher sperm viability (66.6 ± 1.3%) than those frozen with Triladyl® (58.5 ± 1.6%) and Bioxcell® (57.2 ± 2.2%). Acrosome damage of sperm samples frozen using Triladyl® (24.6 ± 1.3%) was significantly higher than when Bioxcell® (17.7 ± 1.7%) and Optixcell® (18.3 ± 1.6%) were used. No significant differences in terms of sperm viability or acrosome integrity were observed after cryopreservation with Andromed® (61.5 ± 1.7% and 19.3 ± 1.7%, respectively) compared to the other three extenders. Regarding ROS generation, Triladyl® showed a better protection against superoxides production (19.98 ± 1.9% HE^+/YoPro-1^-) compared to Optixcell® (27.8 ± 2.8%), Andromed® (33.4 ± 2.5%) and Bioxcell® (43.3 ± 3.4%). Optixcell® (6.7 ± 1%), Andromed® (3.7 ± 2%) and Triladyl® (5.3 ± 1%) triggered similar results when the percentages of viable sperm with a high intracellular H_2O_2 were analized, while this percentage was significantly decreased when Bioxcell® was used as an extender (1.4 ± 0.4%) when compared to Optixcell®. Our results suggest that Optixcell® could be chosen as an egg yolk-free extender for bull sperm cryopreservation due to its better cryoprotective properties.

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Analysis of sperm cell viability and chromatin integrity of ram semen held in a cryopreservation media for 24 hours at 5°C

U.I. Osuagwuh*, P. Cots, M.J. Palomo

Autonomous University of Barcelona, Barcelona, Spain.

Keywords: spermatozoa, nucleoproteins, holding time.

Diluted ram sperm can be held for 24 hours at 5°C prior to cryopreservation or used directly for artificial insemination (AI), however, there is paucity of information on holding semen in a cryopreservation media on viability and most especially the chromatin integrity. This preliminary study was conducted to evaluate the viability and chromatin integrity of ram spermatozoa collected in a favourable reproductive season, and held in a freezing extender for 24 hrs at 5°C. Briefly, ejaculates from 7 males (5 years old) were collected once a week (n = 7) by electro-ejaculation. Despite the general poor quality of the ejaculates, the following mean characteristics were observed: volume (0.68 ± 0.32ml), mass motility (2.02 ± 1.04) and linear motility (67.75 ± 1.27%). Sperm viability was assessed by eosin-nigrosin stain in fresh and refrigerated samples. To assess the chromatin integrity, levels of free cysteine radicals from the disruption of the overall disulfide bonds in sperm head nucleoproteins were determined using the 2, 2-dithiodipyridine technique described in boar sperm by Flores *et al*. (Theriogenology, 76:1450-1464, 2011). Briefly, fresh semen (100 µl) was centrifuged at 5,000 x g for 10 min and the pellet submerged in liquid nitrogen before stored at -80ºC until analysis. Simultaneously, the ejaculate remainder was diluted (1:2) in an extender with 15% (v/v) powdered egg yolk and 5% glycerol in a Tris-based medium and stored at 5ºC at a final concentration of $400x10^6$ sperm/ml. After 24 hours, refrigerated sperm (100µl) was centrifuged in 900µl of PBS at 850 x *g* for 20 min at 4ºC and the pellet stored as above. On analysis, stored samples were resuspended in an ice cold buffer and homogenised through sonication. The homogenates were then centrifuged at 850 x *g* for 20 min at 4ºC, the supernatant was discarded and the pellet resuspended in 500 µl of PBS. These resuspended samples were further diluted (1:100) in a solution of 0.4mM 2,2'-dithiodipyridine and incubated at 37ºC for 1 hour. Levels of free cysteine radicals were determined through spectrophotometric analysis and the results obtained were normalised against the total protein content of the samples determined by Bradford method. General Lineal Model (SPSS 19.0) was used for the statistical analysis and data were presented as mean (mean±SEM). There was no significant difference ($P > 0.05$) among males despite individual male characteristics on parameters studied. Also, no significant difference ($P > 0.05$) was observed between fresh (47.69 ± 4.17%) and 24 hours (46.64 ± 3.89%) sperm samples on viability, but a significant ($P < 0.05$) increase in free cysteine radical levels was observed in the 24 hours refrigerated (6.27 ± 0.59 nmol/µprotein) compared to the fresh (4.84 ± 0.63nmol/µprotein) samples. In conclusion, holding ram sperm collected in a breeding season for 24 hours at 5ºC in a cryopreservation media had a negative effect on chromatin integrity but not on viability of the sperm cells.

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A201E Physiology of Reproduction in Male and Semen Technology

Correlation between conventional sperm tests and chromatin integrity analysis

M.J. Palomo*, P. Cots, U.I. Osuagwuh

Autonomous University of Barcelona, Bellaterra, Spain.

Keywords: sperm, analysis, correlation.

Structural and functional sperm integrity is critical for the capability of spermatozoa to fertilize the egg. The most common tests for assessment of membrane integrity are Eosin/Nigrosin staining (E/N) and Hypoosmotic Swelling Test (HOST). The E/N provides information on sperm membrane structural integrity, while the HOST on sperm membrane functional integrity. However, a wide variety of cell alterations can also reduce sperm fertilising ability as the destabilisation of the sperm chromatin. To assess chromatin integrity, a technique described in boar sperm has been developed by Flores *et al*. [Theriogenology 2011; 76:1450-1464], by determining the levels of free cysteine radicals from the disruption of the overall disulfide bonds in sperm head nucleoproteins using a 2, 2'-dithiodipyridine solution. Therefore, our aim was to evaluate the relationship among these different sperm analysis techniques in order to determine which can better reflect sperm quality. Briefly, an aliquot of fresh semen was diluted (1:20) in a Tris-based medium to assess plasma membrane integrity by E/N and another aliquot was incubated in a hypotonic solution (100 mOsm) at 37°C. After 30 minutes of incubation, two smears per sample were performed by placing 10 mL of sample and 10 mL of E/N on a warm slide, and 200 cells/slide were counted with the aid of an optical microscope at X 1000 magnification. Simultaneously, another aliquot of fresh semen (100 mL) was centrifuged at 5,000 x g for 10 min at 5ºC and the resultant pellet freeze-dried in liquid nitrogen before stored at -80ºC until analysis. Stored samples were resuspended in a cold buffer to be homogenised through sonication. The homogenates were centrifuged at 850 x *g* for 20 min at 4ºC, the supernatant was discarded and the pellet resuspended in 500 µL of PBS. These resuspended samples were then diluted (1:100) in a solution of 0.4 mM 2,2'-dithiodipyridine and incubated at 37ºC for 1 h. Levels of free cysteine radicals were determined using spectrophotometric analysis and the results obtained were normalised against the total protein content of the samples (nmolCys/ug protein) determined by Bradford method. A total of 69 semen samples were collected from 10 different rams and analysed as above. Negative and moderate to high Pearson correlation coefficient was found ($r = -0.506$, $P < 0.0001$) between levels of free cysteine in sperm head nucleoproteins and plasma membrane functional integrity assessed by HOST, while there was no significant relationship between chromatin and plasma membrane structural integrity, even though membrane structural and functional integrity were significantly correlated ($r = 0.685$, $P < 0.0001$). In conclusion, our results suggest that the HOST may provide much more information on sperm quality, being a simple, safe and repeatable method.

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Use of sexed semen for Holstein Friesian cattle breeding in Kazakhstan

A.Y. Serikov[1], T.I. Kuzmina[2], V.P. Terletskiy[2], Y.S. Ussenbekov*[3], A.A. Spanov[3], A.A. Turgumbekov[3], M.S. Gaplaev[4]

[1]Ulsan National Institute of Science and Technology, Ulsan, Republic of Korea; [2]Russian Research Institute of Farm Animal Genetics & Breeding, St.Petersburg-Pushkin, Russia; [3]Kazakh National Agrarian University, Almaty, Kazakhstan; [4]Chechen Research Agricultural Institute, Gikalo, Chechen Republic, Russia.

Keywords: sexed semen, cattle.

Nowadays introduction of innovative techniques is a priority in a modern agriculture. Publications in research journals indicate on economic efficiency only in case when embryo transfer is carried out using top sires as semen donors. Commercial application of sexed semen dates back to 2000. According to the USDA report in 2006-2008 this approach was used on 24,239 cows and 116,846 heifers, conception rate exceeded 90%, whereas pregnancy rate was 27% in cows and 43% in heifers (H.D. Norman & L.J Hutchinson, USDA, 2008). Although sex ratio when using such semen is about 90%, pregnancy rates vary from 25 to 50%. This and high production costs restrict wide use of sexed semen.

The aim of our study was to assess various insemination schemes with frozen/thawed sexed semen of Windsor Manor Zoro bull (511HO8451), Alta Genetics. The insemination was performed with 200 Holstein Friesian heifers that reached live weight of 340 kgs. Four experimental groups of 25, 56, 48 and 71 animals were formed (in 3 - 5 replicates). Hormone treatment was based on injection of PGF2-α (Pfizer) according to the established protocol (two injections of PGF2α with 11 days interval for animals in heat). Insemination in ipsilateral horn was done in first group, heifers in second group were inseminated twice into uterus with 12 hours interval, releasing hormone was injected to animals in the third group, and single insemination into uterus was performed in animals of the fourth group.GnRH was injected immediately prior to artificial insemination (Fertagil, Intervet).Final conception rate in first group was 88% (22 out 25 in total), in second - 89% (50 out of 56 in total), in third - 52% (25 out 48 in total), in fourth -83% (59 out 71 in total). The number of sperm doses per successful insemination was 1.84, 3.3, 1.8, 2.0 in first, second, third, and fourth groups, respectively. It was observed that the highest pregnancy rate was in the second experimental group (insemination twice into uterus, $P < 0.05$, Chi2 test). Nevertheless, high number of sperm doses per successful insemination (3.3) makes this approach economically irrelevant. The approach used in the first group resulted in relatively high value of conception and pregnancy rates and low sperm use (1.84). On the other hand this scheme requires special expertise and experience of inseminator (or use of ultrasound equipment) to find preovulating follicles. The critical point in artificial insemination is to pinpoint cows in heat and right time for insemination. This is particularly important in case of sexed semen which has intrinsically lower quality compared to native one. Three heifers aborted at early stages of pregnancy (up to 4 months) and three heifers died due to heat shock. In remaining 150 calved heifers the sex ratio was 88.6% (133 female calves and 17 male calves).

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A203E Physiology of Reproduction in Male and Semen Technology

Periovulatory secretions modulate *in vitro* boar sperm capacitation decreasing tyrosine phosphorilation

C. Soriano-Úbeda*[1,2], F.A. García-Vázquez[1,2], L. Becerril[3], C. Matás[1,2]

[1]Department of Physiology, Faculty of Veterinary Science, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum", University of Murcia, Murcia, Murcia, Spain; [2]Institute for Biomedical Research of Murcia (IMIB-Arrixaca), Murcia, Murcia, Spain; [3]Laboratory of Animal Reproduction, Universidad Autónoma del Estado de Morelos, Temascaltepec, Estado de Mexico, Mexico.

Keywords: spermatozoa, phosphorylation, periovulatory.

Prior to fertilization the spermatozoa remains attached to oviductal reservoir in a low capacitation status (Suarez, Int J Dev Biol, 52, 5-6, 2008). When ovulation happens the spermatozoa are detached from the oviductal cells and swim toward the oocytes. During this time, the oviductal environment has a higher pH (Rodriguez-Martinez, Theriogenology, 68, 138-146, 2007) and a specific composition that favours the capacitation. The spermatozoa are activated and their tyrosine residues are phosphorylated (Luño, Reproduction, 146 (6), 315-324, 2013). However, under *in vitro* conditions, how sperm capacitation is modulated by oviductal periovulatory factors is still unknown. The aim of this work was to study the tyrosine residues phosphorylation (Tyr-P) (Visconti, Developmental Biology, 214 (2), 429-443, 1999) in spermatozoa capacitated in TALP medium for 180 min at pH 7.4 and 8 supplemented with 1% of oviductal fluid (OF), (Carrasco, Reproduction, 136, 833-842, 2008), and 2% of conditioned medium (CM), formed by oocyte secretions in NCSU-37 after the second stage of *in vitro* maturation (Funahashi, Biol Reprod, 57, 49-53, 1997). Indirect immunofluorescence was performed (Matás, Anim Reprod Sci, 127, 62-72, 2011) in 200 spermatozoa per sample (4 replicates) that were classified in 4 categories according to the capacitation status (Luño, Reproduction, 146 (6), 315-324, 2013): LOW (non-phosphorylated); MEDIUM (equatorial segment phosphorylated); HIGH (equatorial segment and acrosome region phosphorylated); HYPERACTIVATION (flagellum phosphorylated regardless other locations). One-way ANOVA and a Tukey test ($P < 0.05$) were performed. The results (percentage ± SEM) showed that OFCM produced a lower percentage of sperm with a HIGH capacitation status and that was even lower at pH 8 (7.4: 63.9 ± 1.4%^{a}, 7.4OFCM: 54.0 ± 1.4%^{b}, 8: 46.9 ± 1.4%^{c}, 8OFCM: 40.2 ± 1.4%^{d}). At pH 7.4, OFCM produced a higher percentage of sperm with a MEDIUM capacitation status. At pH 8 this percentage was higher but independently of OFCM presence (7.4: 22.3 ± 1.2%^{a}, 7.4OFCM: 29.9 ± 1.3%^{b}, 8: 38.7 ± 1.3%^{c}, 8OFCM: 42.7 ± 1.4%^{c}). The lowest level of HYPERACTIVATION was observed at pH 8 (with or without OFCM) (7.4: 54.0 ± 1.5%^{a}, 7.4OFCM: 48.2 ± 1.4%^{b}, 8: 46.0 ± 1.4%^{b}, 8OFCM: 46.2 ± 1.4%^{b}). Concluding, an alkaline pH and periovulatory oviductal secretions modulate the *in vitro* sperm capacitation maintaining them in a lower status, and it might have a protective effect against premature capacitation.

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A204E Physiology of Reproduction in Male and Semen Technology

Heat shock proteins detection on heat stressed rabbit sperm cells

M. Vendrell*[1,2], L. Ferre-Dolcet[2], M. Sabes-Alsina[1], N. Arcarons[2], R. Morato[2], J. Miro[2], J.-E. Rodriguez-Gil[2], T. Mogas[2], M. Lopez-Bejar[1]

[1]Dpt of Animal Health and Anatomy, Faculty of Veterinary Medicine, Autonomous University of Barcelona, Spain; [2]Dpt of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, Spain.

Keywords: Spermatozoa, Hsp, western blot.

Heat Shock Proteins (HSP) are highly conserved chaperones found in all cell types that have been identified as a defense mechanism for cell survival under severe environmental conditions. Most HSP are constitutively expressed at low levels but some can be up-regulated in response to cellular stresses (physiological, chemical, nutritional or environmental) so to protect cellular proteins against aggregation and denaturation. This study is aimed at determining the presence of HSP60, HSP70 and HSP90 in rabbit spermatozoa exposed to heat stress conditions and to detect if there is any difference between physiological and high temperatures treatments. An up-regulation or an activation of HSP with high temperatures could be a key to induce cryotolerance if heat stress is produced before cryopreservation. Fresh semen samples (6 to 8 New-Zealand White rabbits) were diluted in a semen extender and sperm was incubated for 3h at 4 different temperatures: 32 (scrotal), 37 (body), 42 (heat stress) and 60ºC (killing high temperature). Sperm cells were frozen and kept until the day of processing. Frozen samples were homogenized in 1 mL of protein extraction buffer. Protein concentration of the supernatants was determined by Bradford technique by utilizing a commercial kit. Afterwards, proteins were separated by SDS-PAGE electrophoresis in 10% (w:v) acrylamide gels and transferred to nitrocellulose membranes. Detection of HSP60, HSP70 and HSP90 was performed by using mouse monoclonal anti-HSP60 (1:4000), mouse monoclonal anti-HSP70 (1:1000), mouse monoclonal anti-HSP90 (1:1000), respectively. Membranes were exposed to radiograph films to reveal the HSPs bands (60-90 KDa) after 5 minutes incubation with Luminol Reagent. An goat anti-mouse IgG-HRP was used as secondary antibody at a dilution of 1:2000. Stripping buffer was used to remove the specific HSP marking and a specific anti-mouse a-tubulin antibody for re-test. ImageJ software was used to analyze the adjusted relative densities. Presence of HSP60 and HSP90 was observed in each of the temperatures evaluated. Although an HSP70 band was detectable at 32ºC, 37ºC and 42ºC, no signal for this protein was present at 60ºC. Differences among groups were performed through an ANOVA, followed by the Sidak's post-hoc test fot the HSP analysis. The level of sigificance was set at $P < 0.05$. No significant differences were found between the results. In conclusion, the present study confirms the presence of HSP in rabbit sperm cells as a basal defense system. Further studies about specific location changes and variations in HSP phosphorylation levels could be done so to elucidate if there are changes in protein activity related to high temperatures.

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A205E Physiology of Reproduction in Male and Semen Technology

Follicular and oviductal fluid modulate the protein phosphorylation on serine and threonine residues during boar sperm capacitation

H. Zapata[1,2], E. Paris-Oller[2], F. García-Vázquez[2,3], C. Matás*[2,3]

[1]Laboratory of Biology of Reproduction, Department of Biomedicine, Faculty of Health Sciences, University of Antofagasta, Antofagasta, Chile; [2]Department of Physiology, Faculty of Veterinary Science, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum", University of Murcia, Murcia, Spain; [3]Institute for Biomedical Research of Murcia (IMIB-Arrixaca), Murcia, Spain.

Keywords: Oviduict, follicle, fluid, sperm capacitation, boar.

In boar spermatozoa, protein kinases A have a role in the capacitation and various compounds present in the oviduct could have an important regulatory role during this process. Thus, in this work we evaluated if follicular and oviductal fluids have any influence in PKA activity during sperm capacitation. For this purpose, ejaculated sperm (n=7) was adjusted to 2 x 10^6 sperm/ml in all experimental groups. Each group was incubated for 3 hours in TALP (Rath, J AnimSci, 77,3346–52, 1999) either with or without 1% follicular fluid **(FF),** 1% periovulatory oviductal fluid **(POF)** and 2% cumulus cells secretion media **(MC).** The fluids were collected by aspiration with an automatic pipette as described (Carrasco, Reproduction, 136, 833-842, 2008). The MC was obtained from dishes where groups of 50 COCs had completed the second phase of IVM (with FF but without dbAMPc, PMSG and hCG). The NCSU-37 IVM medium with the COCs was collected and COCs were pipetted to mix COCs secretion with the surrounding media. Centrifugation was the following procedure and the pellet with cellular debris was discarded. After treatment, sperm samples were resuspended in Laemmli buffer and separated on SDS-PAGE. The pattern of protein phosphorylation on Ser and Thr residues was evaluated by western blot. The antibodies used in this study were: phospho-(Ser/Thr) PKA substrate (9624, Cell Signaling Technology, Beverly, USA, 1:2,000) and peroxidase secondary antibody (sc-2004 goat anti-rabbit IgG HRP, Santa Cruz Biotechnology, USA, 1:10,000). Western blot bands were quantified by densitometry (ImageJ software).The results are expressed as densitometric arbitrary units.
The results indicated that sperm incubated with FF and MC have a similar serine and threonine phosphorylation pattern of sperm when incubated in a capacitation media. POF decreases the phosphorylation of these residues during capacitation, as also seen when incubating MC + POF. Concluding, the results suggest that POF regulate the phosphorylation pattern of Ser and Thr residues during sperm capacitation.

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