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Glycosaminoglycans isolated from follicular fluid reduce PKA activity during capacitation in porcine spermatozoa

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Keywords: Glycosaminoglycans, hyaluronic acid, porcine spermatozoa.

Glycosaminoglycans are linear polysaccharides comprised of repeating hexosamine-containing disaccharides that are found in the female genital tract and follicular fluid. Different studies showed that they may be involved in capacitation and acrosome reaction *in vivo* and their presence in sperm reservoir could maintain membrane stability and viability due to their interaction with sperm membrane (Tienthai, Journal of Reproduction and Development, 61, 245-250, 2015). The aim of this study was to investigate the effect of hyaluronic acid (HA) and glycosaminoglycans (GAGs) isolated from folicullar fluid (G-FF) and cumulus oophorus secretions obtained after oocyte porcine *in vitro* maturation (G-COS) on PKA activity and acrosome status after *in vitro* capacitation. Glycosaminoglycans were isolated from COS and FF (from ovaries in the periovulatory phase) by protease digestion, lipid extraction and by different precipitation conditions according to Bellin and Ax (J Dairy Sci 70:1913–1919. 1987). Spermatozoa from five fertile boars (N=5) were incubated for 3 h in TALP (at 38.5ºC and 5% CO_2) or PBS (38.5ºC in air) supplemented or not with G-FF, G-COS or HA (100 ug/mL or 500 ug/mL). PKA activity was assessed by Western Blot using anti-PKA antibody (9624, Cell Signaling Technology, Massachusetts, USA). The relative optical density (R.O.D.) was quantified with ImageQuant TL v8.1 software (GE Healthcare, Life Sciences, Buckinghamshire, UK). Viability was evaluated by propidium iodide 500 mg/mL (Sigma P 4170, Madrid, Spain) and carboxifluorescein 0.46 mg/mL (Sigma D6883, Madrid, Spain), while the acrosome status was analyzed by fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FICT). In each case, 200 spermatozoa were evaluated. Data were analyzed by one-way ANOVA followed by Dunnett´s multiple comparisons test ($P < 0.05$). PKA activity was significantly reduced after the incubation in TALP supplemented with G-FF compared to control (1.74 ± 0.39 and 3.77 ± 0.33, respectively). No effect was observed after the incubation with HA (100 ug/mL or 500 ug/mL) and G-COS. Spermatozoa incubated in TALP showed the lowest percentage (50.83%±3.02) of viability and the highest percentage of acrosomal damage (3.83%±0.54) than spermatozoa incubated in PBS groups (75.66%±1.71 and 1.66±0.40, respectively) ($P<0.05$). However, the addition of HA or GAGs had not effect on this parameter. These results provide evidence that the supplementation of GAGs from FF might prevent sperm capacitation by reducing PKA activity. Nevertheless, GAGs were not able to maintain viability and to protect acrosomal damage of sperm.
Supported by Fundación Séneca, Saavedra Fajardo (20020/SF/16). MINECO-FEDER (AGL 2015-66341-R).

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Effect of Saffron extract on Boujaâd ram semen liquid storage

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Keywords: Boujaâd ram, semen, aqueous saffron extract.

The objective of this study was to evaluate the effect of aqueous saffron extract addition (ASE) (6%) to skim milk and tris on the Boujaâd rams (3-4 years) sperm liquid storage. To achieve this goal, this work was divided into two steps; step 1: Boujaâd ram semen was extended in skim milk based extender supplemented or not with 6% of ASE and stored at 15°C during 24h. While for the step 2: Boujaâd ram semen was extended in Tris egg yolk based extender supplemented or not with 6% of ASE and stored at 5°C during 24h. Ejaculates were collected once a week during 4 weeks for each step using an artificial vagina. Samples were extended to reach a final concentration of $0.8x10^9$ spermatozoa/ml. Then evaluated at different storage times (0 and 24h). A computer-assisted sperm motility analysis (ISAS, version 1.0.17) was used to determine total (TM), progressive motility (PM) and linearity (LIN). Nigrosine-eosin staining, hypo-osmotic swelling test (HOST), and Malondialdehyde (MDA) concentrations were used to determine viability, membrane integrity and lipid peroxidation. The statistical analyses were performed using JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA) program. A factorial design ANOVA analyzed the data of extended semen quality parameters. The statistical model included the addition of saffron extract, and storage periods (0 and 24h). When statistically significant differences were detected, the Tukey's post hoc, was used to compare the means, considering the significance level of $P < 0.05$. Data are expressed as the mean ± SD. In the first step, it was observed that at 0h no significant difference was recorded between the two treatments (Control vs ASE addition) regarding all studied quality parameters: (PM, TM, LIN, viability, HOST and MDA: 79.2±1.66%, 94.25±1.51%, 63.1±3.45%, 95.97±2.89%, 89.41±1.84%, 1.6±0.08 TBARS, nmol/0.810^9 sperm) respectively. While, at 24h it was found that ASE significantly improved the PM (71.23±1.27 vs 74.64±1.25%), TM (91.93±1.2 vs 94.14±1.38%), LIN (61.65±1.83 vs 65±0.83%), viability (92.4±1.47 vs 94.6±0.97%) and HOST (77.9±1.88 vs 81.2±1.08%). As it decreased the MDA production (3.1±0.06 vs 2.6±0.05 TBARS, nmol/0.8 x 10^9 sperm), compared to the control. In the second step; at 0h, the addition of 6% of ASE to the tris eggs yolk extender increased significantly the PM (68.6±3.45 vs 78.6±2.14%). While TM, LIN, viability and HOST, were not affected by this supplementation. At 24h, ASE significantly improved the PM (40.77±3.79 vs 64.67±3.97 %), LIN (28.5±2.93 vs 41.2±1.47 %), viability (89.7±4.55 vs 91.8±2.31%), HOST (65.9±2.93 vs 74.5±1.46%), and decreased MDA production (3.27±0.8 vs 1.81±0.6 TBARS nmol/0.8 x 10^9 sperm) compared to control. While TM was not influenced by the treatments. In conclusion, the addition of the ASE (6%) improved the quality of Boujaâd ram sperm conserved, either at 5°C in Tris egg yolk or at 15°C in skimmed milk based extenders. A biochemical characterization of the aqueous extract and a confirmatory study using artificial insemination are necessary to complete this work.

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Measurements of ram sperm quality under anaerobic and aerobic liquid storage conditions

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Keywords: ram semen, liquid storage, aerobic.

The aim of the present study was to assess sperm quality of INRA180 ram, stored in skim milk extender (SME) at 5°C under aerobic and anaerobic conditions. Ejaculates were collected once a week during 9 weeks from four INRA180 rams, using an artificial vagina. The ejaculates containing spermatozoa with more than70 % total motility and concentrations higher than 3 x 10^9 spermatozoa/ml were pooled. The pool was divided into two parts and each part was extended in SM under aerobic or anaerobic (in sterile syringes) conditions, to reach a final concentration of 0.8 x 10^9 spermatozoa/ml. Samples were evaluated at different storage times; 0, 24, and 48 h. A CASA system was used to determine total motility (TM%) and progressive motility (PM%). Other tests such as nigrosine–eosin staining, Diff-Quick staining, hypo-osmotic swelling test (HOST), and Malondialdehyde (MDA) concentrations were used to determine viability(%), morphology (%), membrane integrity (%) and sperm lipid peroxidation. The statistical analyses were performed using JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA) program. A factorial design ANOVA analyzed the data of extended semen quality parameters. The statistical model included the fixed effect of storage conduction (aerobic vs anaerobic), and storage periods (0, 24 and 48 h). When statistically significant differences were detected, the Tukey's post hoc, was used to compare the means and standard errors, considering the significance level of $P < 0.05$. Data are expressed as the mean ± SE.The results of PM (71.72±1.34%), TM (90.78 ± 1.03%), viability (97.5±0.52%), abnormality (2.44±0.21%) and membrane integrity (94.5±0.84%), showed that, at 0h, there was no difference between the two storage conditions (anaerobic vs aerobic). However, lipid peroxidation was significantly higher in aerobic condition(0.59±0.03 TBARS, nmol/10^8 sperm) compared to the anaerobic one (0.47±0.03 TBARS, nmol/10^8 sperm). At 24 h, semen stored in the anaerobic condition shows the highest PM (64.22±1.17 vs 53.88±2.31%), membrane integrity (88.44± 0.68 vs 84.22±0.95%) and the lowest lipid peroxidation (1.32±0.01 vs 2.05±0.08 TBARS, nmol/10^8 sperm) compared to the aerobic storage ($P<0.05$). While the TM (80.72±1.22%), viability (91.14±0.91%) and abnormality (6.25±0.31%) were not affected by the storage condition ($P>0.05$). At 48 h, the best semen quality results were obtained in anaerobic condition. And that concerned; PM (50.66±1.75 vs 33.66±1.12%), viability (81±0.46 vs 76.22±1.31%), abnormality (10.5±0.32 vs 13.05±0.52%), membrane integrity (63±1.49 vs50.44±1.12%) and lipid peroxidation(1.35±0.03 vs 3.56±0.09 TBARS, nmol/10^8 sperm) compared to aerobic one. Whereas, for the TM (74.28 ±2.21%) is was not affected by the two treatments. In conclusion, INRA180 ram semen stored at 5°C in a skim milk-based extender exhibited highest quality parameters under anaerobic exposure compared to aerobic exposure.

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Cyclin/Cdk complexes are involved in control of actin dynamics during boar sperm capacitation

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Keywords: sperm capacitation, Cyclin/Cdk complexes, Aminopurvalanol A.

Mammalian spermatozoa are virtually infertile immediately after ejaculation and will only reach their full fertilizing ability after they reside within the female genital tract for hours to days, depending on the species. This process of capacitation implies marked changes in the whole biochemical machinery expressed by spermatozoa. Thanks to the adoption of high-throughput technologies (Chronowska, *Biomed Res. Int.* vol 2014 (2014)) it was demonstrated that male gametes express proteins involved in cell cycle control, that are thought to be not present or active in sperm cells (Hydbring et al., *Nat. Rev. Mol. Cell Biol.* 17, 280–292, (2016)). To identify the cell cycle proteins potentially involved in sperm capacitation by a computational modelling approach and to assess their actual role in vitro capacitation and IVF by inhibiting these with a potent and specific inhibitor of the identified proteins, Aminopurvalanol A (AA). Cell cycle network was created and analysed using Cytoscape 3.3.0, by previously using the pathway database Reactome as data source. All the chemicals were purchased by Sigma Aldrich and were of the purest analytical grade. Semen samples were processed using a validated protocol (Barboni et al., *PLoS One* **6**, e23038 (2011)) and spermatozoa were incubated under capacitating conditions with or without AA at different concentrations (20, 10 and 2 μM) during 4 hours. Then, acrosome integrity (PSA staining), actin polymerization (Phalloidin staining), tubulin relocation (immunocytochemistry assays), membrane lipid remodelling (FRAP), and fertilizing ability (IVF) were evaluated in vitro. The network representing the molecules involved in cell cycle control was created by using an in silico approach (Bernabò et al., *OMICS* 19, 712–21, (2015); Bernabò et al., *BMC Syst. Biol.* **5**, 47, (2011a)). Among the whole proteins involved in cell cycle, it was possible to highlight the central role of Cyclins/Cdk in signal transduction during capacitation. With this information, in vitro experiments were performed to confirm the finding. By adding the Cyclins/Cdk inhibitor AA at different concentrations (20,10 and 2 μM) during capacitation it was possible to evince a dose-dependent inhibition of actin polymerization (phalloiding staining), with the consequent loss of acrosomes (PSA staining) and a decrease of in vitro fertilizing ability of spermatozoa (IVF), far-reached with the highest concentrations of AA ($p<0.05$). Otherwise, AA showed not to interfer with membrane lipid remodelling (FRAP analysis, DILC12 staining) or cytoskeleton tubulin dynamics (immnunocytochemistry assays). Cyclin/cdk complexes could be a new element in control system of actin polymerization during boar sperm capacitation. This data could revamp the knowledge on biochemistry of capacitation and could suggest new perspectives in studying male infertility. Marina Ramal Sanchez is granted by Marie Sklodowska-Curie ITN REP-BIOTECH 675526, European Joint Doctorate in Biology and Technology of the Reproductive Health.

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Variation of melatonin, testosterone and antioxidant enzymes in seminal plasma of three ram breeds under tropical conditions

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Keywords: Ram seasonality, rainy season, drought season.

In temperate regions, pineal melatonin and photoperiod regulates sheep seasonality (Chemineau *et al. Reprod Dom Anim* 43 (Suppl. 2), 40–47. 2008). In tropical conditions, with equal duration of day light, small ruminant reproduction is regulated by the annual cycle of rainfall and food availability more than photoperiodic changes (Morales *et al*. *Small Rum Res* 137, 9-19. 2016). Therefore, melatonin must be playing other functions, like the regulation of the antioxidant defense system (Mayo *et al. Cell Mol Life Sci.* 59, 1706–1713. 2002). Although melatonin, testosterone and antioxidant enzymes (AE) are present in the ram seminal plasma of seasonal breeds (Casao, *et al. Reprod Biol Endocrinol* 8, 59. 2010), there is no information on hormonal concentration and AE activity in the ram seminal plasma from tropical regions such as Colombia, located at equatorial level, with a bimodal regime of rain in the Andean region. Thus, the aim of this study was to evaluate the variation of melatonin, testosterone and AE in the seminal plasma form three sheep breeds (Colombian Creole, Romney Marsh and Hampshire) under tropical conditions. Semen from twelve rams (four rams from each breed) was collected weekly for one year by artificial vagina. Sires were housed at the National University of Colombia, located in Mosquera (4°40'57'' N. 74°12'50'' W) at 2510 m above the sea level. Seminal plasma was extracted by double centrifugation at 9000xg for 10 min at 4ºC, filtered through a 0,22 µm Millipore membrane (Merck, Darmstadt, Germany) and kept at -20 ºC until use. Melatonin and testosterone concentration were measured by a commercial competitive immunoassay (Direct saliva melatonin ELISA kit, Bühlmann Laboratories AG, Switzerland and Testosterone-ELISA, DiaSource InmunoAssays S.A., Belgium), following the manufacturer's instructions. The activity of catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRD) were analyzed as previously described (Casao *et al. Anim Reprod Sci* 138, 168–174. 2013). Results were grouped into four environmental seasons; two rainy (March-May, September-November) and two dry (December-February, June-August) and analyzed by two-way ANOVA followed by Bonferroni post-test (GraphPad Software, La Jolla, CA, USA). Melatonin concentration was lower ($P<0.05$) in the March-May rainy season (25.8±0.7 pg/mL) than in the other seasons (32.5±1.5 pg/mL for Sep-Nov, 35.2±1.7 pg/mL for Dec-Feb and 36.3±1.8 pg/mL for Jun-Aug). Testosterone showed higher concentrations ($P<0.05$) in Romney Marsh and Hampshire breeds than in Creole (3.4±0.1 and 3.5±0.2 *vs*. 2.3±0.3 ng/mL) during the June-August dry season. GPx activity were higher ($P<0.05$) between Sep-Nov (10.4±0.9 nmol/min.mL) and Jun-Aug (10.6.±1.3 nmol/min.mL) compared with Dec-Feb (5.2±0.9 nmol/min.mL) and Mar-May (5.4±1.1 nmol/min.mL). In conclusion, melatonin, testosterone and antioxidant enzymes are present in seminal plasma of rams under tropical conditions, and show seasonal or breed differences. Grants: Colombia 110157635854 and Col576-2012, Spain AGL2014-57863-R and AGL2013-43328-P.

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Effects of short exposure of bull semen to Roundup? on sperm kinetics and on *in vitro* embryo production

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Keywords: Roundup?, semen, embryo.

Common toxicants, such as herbicides, are considered as potential threats for fertility. The aim of the present study was to investigate the impact of a low concentration of the herbicide Roundup® (1ppm of Roundup®, -Monsanto-corresponding to 360ng/ml of the active ingredient, glyphosate), on sperm motility of frozen-thawed bull semen and on the subsequent *in vitro* bovine embryo production. In experiment 1, frozen semen samples, from the same bull and ejaculation, were allotted in two groups [treated (R) and controls (C), each n=3, in three replicates)] and separated by swim–up. In group R, the swim-up medium was modified with the addition of 1ppm of Roundup®. After 1 hour, all semen samples were centrifuged for 5 min at 10000r/min, the supernatant was discharged, 800 μl of new swim-up medium was used to reconstitute the semen pellet, and samples were evaluated for kinetics by CASA [(progressive, immotile, rapid, medium, slow moving spermatozoa, curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), linearity (LIN), straightness (STR), beat cross-frequency (BCF), amplitude of lateral head displacement (ALH) and wobble (WOB)]. In experiment 2, immature cumulus oocyte complexes (COCs n=494, 4 replicates) were obtained by aspirating small-medium size follicles (2-6mm) from ovaries of slaughtered cows. COCs were matured in TCM-199 supplemented with 10% (v/v) FCS and 10 ng/ml EGF at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. Matured oocytes were divided in two groups, (control (C) n=162 and treated (R) n=332) and were inseminated using frozen-thawed swim-up separated bull sperm (as in exp 1) at a concentration of 1 x 10^6 spermatozoa/ml. Treated sperm was exposed to the herbicide only for 1 hour; this was during the swim-up process. Gametes were co-incubated at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. At approximately 20 h post insemination (hpi), presumptive zygotes were denuded, transferred to 25 ml culture droplets under mineral oil, and cultured in SOF supplemented with 5% (v/v) FCS at 39°C in 5% CO_2, 5% O_2 and max. humidity. Cleavage and blastocyst formation rate were evaluated at 48 hpi and on days 7, 8 and 9 respectively. Comparisons on semen kinetics were analyzed by t-test, while cleavage and blastocysts formation rates were carried out by χ^2; in all cases significance was set at 0.05 level. Sperm evaluation by CASA revealed no difference in any of the parameters studied. Cleavage rate was similar between groups (C: 88.3%, R: 90,9%, p=0.5) while in group R blastocyst formation rates were steadily lower than that of group C (day 7, C: 24.7 ± 3.6% , R:14.5±4.8%; day 8 C:29.7±3.5%, R:18.1±5.8%; day 9 C: 32.8±3.5% , R:18.1±5.8%; in all cases $p<0.02$). These results imply that short exposure to roundup brings about alterations to sperm that are expressed during early embryo development. Further research is underway to evaluate sperm of DNA integrity and to assess embryo quality.

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Evaluation of exogenous DNA integration and fertilization ability of transfected boar spermatozoa

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Keywords: sperm, transfection, embryo.

The use of boar spermatozoa as vectors for introduction of exogenous DNA into the oocyte during fertilization could be an alternative and simple method for generation of transgenic pigs. So far, the results of using sperm as DNA vectors are very controversial. The aim of this study was to evaluate the integration of gene construct and the ability of fertilization by transfected boar spermatozoa. The boar sperm was transfected by direct incubation with the p12hGH-GFPBsd gene construct labeled with rhodamine. We investigated sperm of 92 ejaculates collected from 30 boars of which 16 ejaculates from 4 boars were selected for transfection. Sperm motility was evaluated after centrifugation and transfection. The presence of the gene construct in transfected boar spermatozoa was detected by fluorescence *in situ* hybridization (FISH) by assessment of a positive fluorescence signal for rhodamine. The control group consisted of non-transfected boar spermatozoa hybridized with labeled construct. Boar sperm after transfection was used for *in vitro* fertilization of pig oocytes. For this purpose, sperm motility was evaluated before and after capacitation. The control group consisted of capacitated, non- transfected sperm. Selected semen from 3 boars after transfection and capacitation, exhibiting the best motility parameters was used for *in vitro* fertilization (IVF) of oocytes. Presumptive zygotes were cultured in the NCSU-23 medium up to the blastocyst stage. The percentage of potential cleaved zygotes, morulae and blastocysts was evaluated and the presence of the gene construct in embryos was assessed by observing a positive fluorescence signal for rhodamine. After transfection a decrease of sperm motility was observed. The gene construct was detected in 7138 (47.1%) out of 15981 analyzed transfected spermatozoa. Two types of transgene fluorescence were observed: a single fluorescence signal specific to a given transgene, and a fluorescence signal indicating transgene-coated sperm. Among the control group, the signal was present in 2082 (15.3%) analysed cells, which should be regarded as a false-positive result obtained from the fluorochrome attached to the probe. After capacitation of transfected boar sperm, slight differences in seminal motility were observed in comparison to the control group. As a result of IVF with transfected sperm, out of 77 (59.68%) presumptive zygotes 19 (24.64%) and 9 (11.68%) developed to the morula and blastocyst stage, respectively. The development of the other presumptive zygotes have been stopped at the stage of 3-4 blastomers. The presence of the gene construct in evaluated embryos was not detected. Our results confirm the ability of boar transfected spermatozoa to bind exogenous DNA. However, in embryos obtained after in vitro fertilization with DNA transfected spermatozoa no gene expression was detected. Further studies are required to determine the transfection efficiency using ICSI technique.
Supported by Fund of Own Research of IZ PIB no. 07-2.05.7 (2015-2016).

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Effect of foodborne contaminants on sperm fertilization competence and embryonic development

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Keywords: AFB1, ATZ, sperm.

There is growing concern about the effects of environmental toxins on human and domestic animal reproduction. Among the toxin sources, food products can potentially be contaminated with mycotoxins and/or pesticides. Aflatoxins are poisonous by-products of the soilborne fungus *Aspergillus*, found in food products such as maize, rice and wheat. Of these, aflatoxin B1 (AFB1) is the most toxic. Atrazine (ATZ) is an herbicide, that is extensively used to control weeds in broadleaf and grassy crops. ATZ is considered a ubiquitous environmental contaminant and is frequently detected in the ground, surface water and various types of crops. In the body, ATZ is metabolized to diaminochlorotriazine (DACT), which is further detected in the urine, serum and tissues. Exposure to ATZ and DACT impaired sperm viability, acrosome reaction and mitochondrial function (Komsky-Elbaz, Reprod Toxicol 67:15, 2017). Furthermore, exposure to AFB1, ATZ or DACT resulted in DNA fragmentation in sperm. However, these toxins' effects on fertilization have never been examined. Examine the effects of AFB1, ATZ and DACT on sperm fertilization competence and early embryonic development. Cumulus oocyte complexes (n=30–60/group; 6 replicates) were aspirated from bovine ovaries, in-vitro matured (22 h) and fertilized (18 h) with fresh semen. Before fertilization, sperm was capacitated in the absence (control) or presence of AFB1 (10 μM; Cayman Chemical, MI, USA), ATZ (0.1 or 1 μM) or DACT (1 or 10 μM; Chem Service Inc., PA, USA). Cleavage into 2- to 4-cell-stage embryos and blastocyst-formation rates were evaluated 42 h and 7 days post-fertilization, respectively, using JMP-7 software (SAS Institute Inc., 2004, Cary, NC, USA). Cleavage and blastocyst-formation rates, and distribution of blastocysts to various developmental stages were compared by chi-square test followed by Fisher's exact test. Data are presented as means. Exposing sperm to 10 μM AFB1 pre-fertilization reduced the proportion of embryos that cleaved to the 2- to 4-cell stage relative to controls (70.9 vs. 85.1%). Similar findings were noted when sperm was exposed to 1 μM ATZ (60.7 vs. 91.3%), 1 μM DACT (72.6 vs. 85.1%), or 10 μM DACT (64.6 vs. 85.1%) relative to controls, respectively ($P < 0.005$). Blastocyst-formation rate was significantly lower when oocytes were fertilized with sperm exposed to ATZ (0.1 or 1 μM; 7.7 and 8.7%, respectively) or DACT (1 or 10 μM; 20.5 and 16.6%, respectively) relative to controls (30.4%; $P < 0.04$). Blastocyst-formation rate did not differ from controls after fertilization with sperm exposed to AFB1. Distribution into different embryonic stages differed among groups, with significantly lower rates of development to the blastocyst stage for ATZ-treated sperm (0.1 or 1 μM; 6.6 and 8.7%, respectively) relative to controls (16.1%; $P < 0.05$). The findings reveal risk associated with exposure of sperm to foodborne contaminants. Even relatively low doses of AFB1, ATZ or DACT and short time exposure impair fertilization and blastocyst formation.

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Heat stress effects on reactive oxygen species production and lipid peroxidation in bovine spermatozoa

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Keywords: oxidative stress, semen analysis, heat stress.

The aim of this study is to elucidate the effects of heat stress on oxidative status in bovine spermatozoa by quantifying reactive oxygen species (ROS) and lipid peroxidation (LPO). Heat stressed (HS) and non-heat stressed (NHS) frozen bovine semen samples were examined. HS semen, collected 14 to 42 days after artificial scrotal insulation, showed lower protamination and motility, and changes in the methylation of paternal pronuclei (Rahman et al., Theriogenology, 76, 1246–1257, 2011). Spermatozoa were passed through a discontinuous Percoll gradient (45/90% (v/v); VWR International) and adjusted to a final concentration of $2.5x10^6$ cells/ml in PBS. 100mM (final concentration) of 2′,7′-Dichlorofluorescin diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, USA) was added to the sperm samples to stain ROS and incubated at 37ºC for 15 minutes. Dead cells were stained with 1.5µM (final concentration) of propidium iodide (PI) and analyzed using a Cytoflex flow cytometer (Beckman Coulter, Brea, USA) (n=4). 10µM (final concentration) of BODIPY 581/591 C11 (Thermo Fisher, Waltham, USA) was added and incubated at 37ºC for 15 minutes before Cytoflex analysis (n=4). It emits red fluorescence in the non-oxidized state, shifting to orange and green after LPO. 5mM (final concentration) of Luminol sodium salt (Sigma-Aldrich, St. Louis, USA) was added to measure chemiluminescence for 15 and 30 minutes at 37ºC using a luminometer (n=3). PI, DCFH-DA and BODIPY signals were acquired screening a minimum of 5000 spermatozoa per sample. Data were analyzed using the Student's t-test ($p \leq 0.05$) and Spearman correlation. A significantly higher percentage of PI^+ dead cells was present in HS (30.7%) than in NHS semen (21.9%). No differences were observed in the percentage of DCFH-DA^+ cells between HS and NHS semen. However, a higher mean fluorescence intensity (MFI) was observed in HS compared to NHS semen. Significant differences were observed in BODIPY between red stained HS (83.6%) and NHS (91.3%) sperm cells, although no significant differences were observed in the percentage of green stained cells. This shift towards green fluorescence was higher in HS compared to NHS semen. Although no significantly higher ROS production in HS compared to NHS semen was observed after luminol quantification, there was a positive correlation between ROS production (luminol) and LPO (BODIPY green) ($r=0.82$, $p=0.01$).
The survival rate of sperm cells was higher in NHS than in HS semen, while a higher LPO and ROS production were observed in HS compared to NHS semen. Further evaluations are needed to better understand these effects of heat stress on the oxidative status of bovine spermatozoa.

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Effect of antioxidants and thawing rates on the quality of cryopreserved camel sperm

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Keywords: camel sperm, cryopreservation, antioxidants.

The viscous seminal plasma of camel semen may prevent penetration of cryoprotectants into spermatozoa, making cryopreservation difficult. This study examined whether addition of antioxidants catalase (CAT), carnitine (CARN) and glutathione (GSH) pre-freezing could improve post-thaw semen quality, and/or increasing thawing temperature from 30°C (30 sec) to 60°C (10 sec). Two ejaculates were collected from each of four fertile dromedary males. Spermatozoa were separated from seminal plasma by single layer centrifugation (SLC) and sperm pellets were resuspended in freezing extender supplemented with individual antioxidants or control without antioxidants for freezing in liquid nitrogen. One straw from each treatment was thawed at 60°C for 10 sec and the other at 30°C for 30 sec; sperm quality was evaluated at 0, 1 and 2h post thawing. Parameters evaluated were total motility (TM), progressive motility (PM) and kinematics: ALH (lateral head displacement), BCF (beat cross frequency), LIN (linearity), STR (straightness), VAP (average path velocity), VCL (curvilinear velocity), VSL (straight line velocity) using computer-assisted semen analysis, membrane integrity and acrosome integrity (eosin-nigrosin), and membrane functionality (HOST-test). Normality of the samples was analysed by Saphiro-Wilk test. Normally distributed samples were analysed using ANOVA (Antioxidants) or t-Student (Thawing rates). Non-normally distributed data were analysed using Kruskal-Wallis (Antioxidants) or Mann-Whitney (Thawing rates). Values are means ± SEM. There were no differences among treatments at 0h for any parameters. A significantly higher TM was observed at 1h post thawing (P=0.009) for CAT (37%) and CARN (32%) compared to control (26%). After 2 h, TM was significantly higher (P=0.001) for CAT, CARN and GSH (27%, 25%, 23%, respectively) compared to control (15%). VAP 1h post thawing was increased for CAT (72 µm/s) and CARN (67 µm/s) compared to control (62 µm/s) (P=0.014). At 2h post thawing the following parameters were significantly higher for CAT, GSH and CARN: PM (9%, 11%, 9%, respectively; P=0.004), ALH (7 µm for each; P=0.015), VAP (65 µm/s, 66 µm/s, 62 µm/s; P=0.014), VCL (136 µm/s, 139 µm/s, 134 µm/s; P=0.019), VSL (43 µm/s, 46 µm/s, 42 µm/s; P=0.013) compared to control (4%, 6 µm, 54 µm/s, 117 µm/s, 36 µm/s, respectively).
Thawing at 60°C for 10 sec gave significantly higher TM at all time points (P<0.01), 40%, 35%, 26% at 0, 1 and 2 h compared to 37ºC for 30 sec (34%, 29%, 19%, respectively). Greater values were also observed at 60ºC for PM at 0 h (18%; P=0.002), 1h (P=0.007; 16%) and 2h (10%; P=0.013) compared to 37ºC (13%, 12%, 7% respectively). Thawing at 60º C increased the values of ALH (7.9 µm; p=0.033), STR (68%; P=0.034) and VCL (149 µm/s; P=0.004) at 1h compared to 37ºC (7.6 µm, 67% and 137 µm/s, respectively). No differences were observed in membrane or acrosome status for the different thawing rates.These results suggest that antioxidants exert a protective effect during cryopreservation of camel spermatozoa; it is better to thaw camel semen at 60°C for 10 sec compared to 37°C for 30 sec.

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Mouflon *(Ovis musimon)* sperm cryosurvival is better at the end of the rutting season coinciding with low plasma testosterone concentrations

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Keywords: semen, cryopreservation, seasonality.

Cryosurvival of spermatozoa in Iberian ibex (*Capra pyrenaica*) is poorer at the onset and in the middle of the rutting season, when plasma testosterone levels are the highest, than at the end of the rutting season coinciding with fall of testosterones levels. We hypothesized that high plasma testosterone concentration might have a negative effect on sperm cryosurvival, and thus a similar situation may be found in other wild ruminants, such as the mouflon (*Ovis musimon*). Sperm samples were obtained from 22 mouflons, using the transrectal ultrasound-guided massage of the accessory sex glands technique. Samples were collected during autumn (October) when plasma testosterone concentrations are high, and at the end of the rutting season (January), when levels of testosterone tend to decrease to basal levels. Sperm motility was assessed with a computer-aided sperm analysis system. Membrane integrity and acrosomal status were evaluated by fluorescence and by the eosin-nigrosin technique. Morphological abnormalities and acrosome integrity were evaluated in samples fixed in buffered 2% glutaraldehyde. Each sample was cryopreserved following two different protocols. Ejaculates were diluted using a Tris-TES-glucose-based medium with 6% egg yolk and two different cryoprotectants: glycerol 5% for the traditional freezing protocol in straws in liquid nitrogen vapors (frozen sperm), and sucrose 100 mM for the ultrarapid freezing protocol in pellets (vitrified sperm). Plasma testosterone concentrations were measured by radioimmunoassay. Statistical analysis was performed by one-way ANOVA. Sperm quality of frozen-thawed and vitrified-warmed samples was higher in January, when levels of testosterone are decreasing, than in October. Plasma testosterone concentration was higher ($P<0.01$) in October (5.49 ± 1.33 ng/ml) than in January (1.02 ± 0.55 ng/ml). There were no differences in fresh sperm variables between samples collected in October and in January. Frozen-thawed sperm cryopreserved in January had a total sperm motility, curvilinear velocity (VCL), average path velocity (VAP), amplitude of lateral head displacement and beat-cross frequency greater than samples collected in October ($P<0.05$). Sperm viability was also higher in frozen-thawed samples collected in January than in October ($P<0.05$). Vitrified-warmed sperm had a VCL ($P<0.01$), VAP ($P<0.01$), straight-line velocity ($P<0.05$) and viability ($P<0.05$) higher in samples collected in January than in October. These results confirmed the hypothesis that the pick of plasma testosterone concentration that occurs in October, could affect negatively to mouflon sperm cryosurvival.

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Melatonin has a protective role against cryocapacitation of ram spermatozoa

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Keywords: melatonin, sperm cryopreservation, apoptosis.

Melatonin is present in the ram seminal plasma and in the male reproductive tract. In vitro treatments with melatonin have shown a direct effect on ram spermatozoa by decreasing apoptosis-like changes, affecting capacitation, and increasing the IVF results. Therefore, this study raises the hypothesis that melatonin could have a protective effect against cold-shock in ram spermatozoa, avoiding changes related to cryocapacitation such as the increase in apoptosis indicators that sperm suffer at low temperatures. Briefly, two successive ejaculates were collected three times per week (from February until June) from nine healthy *Rasa Aragonesa* rams (2-6 years old) of proven fertility with the aid of an artificial vagina. Second ejaculates were pooled and processed together in order to eliminate individual differences. After sperm selection by a swim-up method, aliquots of 300 µl were incubated with 100 pM, 10 nM and 1 µM melatonin (Sigma-Aldrich Co., dissolved in PBS with 0.1 ‰ DMSO) for 30 min at room temperature. Then, samples with melatonin plus a control sample with 0.1 ‰ DMSO were directly cooled at 5 °C on ice-bath for 10 min followed by 5 min at 37 °C (cold-shock treatment). The following sperm functionality parameters were analyzed in the swim-up sample and in cooled samples (with and without melatonin): motility, using a CASA system (ISAS 1.04; Proiser SL, Valencia, Spain); membrane integrity (CFDA/PI stain); capacitation state related to intracellular calcium distribution by chlorotetracycline (CTC) staining, and tyrosine phosphorylation of membrane proteins by SDS-PAGE immunoblotting. The apoptotic markers assessed were phosphatidylserine (PS) translocation (Annexin V/PI stain); DNA damage by TUNEL (In situ cell death detection kit, ROCHE), and caspase activity (Vibrant® FAM™ Caspase-3 and -7 Assay, Invitrogen). Eight replicates were performed and data were compared by Chi-squared test. The obtained results indicate that cold-shock produced a significant ($P<0.001$) decrease in all the seminal quality parameters, except in DNA damage. The addition of 100 pM and 10 nM melatonin before cooling decreased significantly ($P<0.05$) the percentage of capacitated spermatozoa (51.71% ±2.55 and 49.86% ±2.83 respectively) comparing to the control sample (57.43% ±2.88). The lowest dose of melatonin (100 pM) reduced the PS translocation and caspase activation compared to the control sample without hormone. The addition of melatonin did not result in significant differences in motility, membrane integrity or tyrosine phosphorylation. It can be concluded that melatonin at low doses (100 pM) is able to prevent, at least partially, the cold-associated apoptosis and premature capacitation-related changes. Grants: CICYT AGL 2014-57863-R and DGA 2016-A26 FSE.

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Effect of seminal plasma on cytokine production from bovine endometrial epithelial cells in culture

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Keywords: bovine seminal plasma, uterine cell, cytokine response.

Seminal plasma (SP) is involved with immune-regulation in the female reproductive tract through specific cytokines. Variations in fertility among bulls could be due to SP. The objective was to investigate the type and level of cytokine response bovine endometrial epithelial cells (bEEC) in culture (passage 5) after challenge with SP. Donor bulls were categorized as below average (L) or above average fertility (H) according to an index based on the 56-day non-return rate from at least 1,000 artificial inseminations. Bulls of average fertility scored 100. The L-bulls had a score of ≤ 92 (n=2) and H-bulls > 104 (n=3). Approximately 5 – 13 x 10^5 bEECs per flask were challenged with 1% or 4% SP from L- or H-fertility bulls (L1, L4, H1, H4, respectively) or 1% or 4% PBS as control (C1, C4) in 13 replications with cells from 8 uteri. After 72h, the total number of cells, stained with trypan blue, was counted in a Burker hemocytometer. The supernatant was analysed for transforming growth factor beta (TGF-β1, TGF-β2 and TGF-β3) by Luminex (MILLIPLEX™ MAP, Merck Millipore, USA) and Interleukin 8 (IL-8) by ELISA (Bovine IL-8, MABTECH, Sweden). The concentration of each cytokine was calculated (pg/million cells). Data were analysed using the mixed model in SAS® (Proc Mixed, SAS® 9.3, USA). Fertility of bull, concentration of SP, and their interaction were fixed parts of the model, with cytokine response as variable parameter. Cow and cow interaction with replication were used as random factors. Post-hoc comparisons were adjusted for multiplicity using Tukey's, and the Contrast option was used to analyze individual differences. All values are presented as LSMEAN ± SEM. Challenge had significant effects on cytokine production (TGF- β1, TGF- β2 and IL-8) due to fertility of bull ($p<0.0001$), concentration of SP ($p<0.0001$) and the interaction between both factors ($p<0.0001$). There were no differences in TGF- β1, TGF- β2 and IL-8 production after challenge with L1 (4.8±3.2, 52.2±19.7, 18.0±5.1; x10^3 respectively) and H1 (7.1±3.0, 38.9±19.0, 18.4±4.7 x10^3 respectively) compared to C1 (3.5±3.0, 27.1±18.9, 17.5±4.7 x10^3 respectively) and C4 (3.2±3.1, 25.3±19.5, 17.1±4.9 x10^3 respectively). A higher production of TGF-β1, TGF-β2 and IL-8 ($p<0.0001$) resulted from challenge with L4 (20.6±3.1, 136.3±20.5, 54.8±4.9 x10^3 respectively) or H4 (18.6±3.2, 106.6±19.9, 44.6±5.1 x10^3, respectively); challenge with L4 SP was differed from H4 ($p<0.05$). For TGF-β3, fertility of bull ($p<0.05$), concentration of SP ($p>0.05$) and the interaction between factors were significant ($p<0.01$). The highest production of TGF-β3 was found in L4 (2.4±0.4 x10^3) than H4 (1.2±0.4 x10^3) ($p<0.05$), L1 (0.6±0.4 x10^3) ($p<0.01$), H1 (0.4±0.4 x10^3) ($p<0.01$), also C1 (0.5±0.4 x10^3) and C4 (0.2±0.4 x10^3) ($p<0.01$). In conclusion, higher concentrations of SP stimulated more cytokine production; 4% SP from L-bulls stimulated more TGF-β1, TGF-β2, TGF-β3 and IL-8 production than SP from H-bulls, which could be associated with impaired cell adhesion or cell damage.

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Comparison of the apoptotic like-changes in boar semen before and after in vitro capacitation

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Keywords: boar, apoptosis, spermatozoa.

Boar semen is very sensitive to all biotechnical modifications, including *in vitro* capacitation and *in vitro* fertilization. Moreover, the quality of boar semen may affect the efficiency of *in vitro* fertilization. However, the process of capacitation required by spermatozoa to acquire the fertilization ability is impaired in the IVF system. Presence of the apoptotic-like changes (ALC) in capacitated spermatozoa can lead to decreased fertility. The assessment of ALC can be useful for estimating sperm ability for fertilization. The purpose of this study was to determine the ALC in boar spermatozoa before and after *in vitro* capacitation. Semen was obtained from 11 boars of different breeds with normal fertility from the AI Center in Klecza Dolna. The sperm capacitation took place during incubation in a medium based on TCM-199 (Sigma, Germany) for 1 hour, at 39^{o}C and 5% CO_2 in the air. For assessment of ALC, semen was incubated with the fluorophore YO-PRO-1 (Vybrant Apoptosis Assay Kit≠4, Molecular Probes, USA) (Trzcinska et al, Anim Reprod Sci, 124, p 90-97, 2011) in the dark for 20 – 30 minutes, in room temperature before and after *in vitro* capacitation. After incubation, the semen was analysed under a fluorescent microscope. Statistical analysis was performed using the t-test. The mean percentage of viable spermatozoa before capacitation ranged from 65 to 91% (mean 78.4%; P>0.01), while after capacitation from 44 to 88% (mean 61.6%; P>0.01). The viable spermatozoa with ALC before capacitation oscillated between 0.5 and 9% (mean 4.4%) and after capacitation 0 and 21% (mean 5.9%). The percentage of nonviable spermatozoa before capacitation ranged from 4 to 32% (mean 16.5%; P>0.01), while after capacitation from 8 to 48% (mean 32.6%; P>0.01). When boar semen before *in vitro* capacitation was compared with semen after capacitation, an increase in the percentage of cells with ALC was detected accompanied by a significant decrease in nonviable spermatozoa. In conclusion in vitro capacitation of boar semen resulted in an increase in the percentage of apoptotic-like changes which may result in a decrease in fertility.
Supported by Fund of Own Research of IZ PIB no. 07-2.06.7 (2015-2017).

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Progesterone-induced changes in the ram sperm acrosome reaction are inhibited by the antagonist mifepristone

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Keywords: mifepristone, acrosome reaction, ram spermatozoa.

Progesterone (P4) is an important hormone regulating the reproductive functions and may exert rapid non-genomic effects on sperm functionality. P4 may stimulate sperm hyperactivation, chemotaxis, in vitro capacitation and the acrosome reaction in several species. Most of these rapid actions could be mediated by P4 binding to membrane receptors on the sperm surfaces. The aim of this study was to investigate the effect of progesterone on ram sperm functionality and to elucidate whether these actions are mediated by its binding to sperm membrane receptors. For this purpose, sperm samples were incubated in capacitating conditions in the presence of P4 with or without mifepristone, a progesterone receptor antagonist. Briefly, two successive ejaculates were collected three times per week from nine males (2-6 years old) with the aid of an artificial vagina, and second ejaculates were pooled and processed together in order to eliminate individual differences. After sperm selection by a swim-up-dextran method, samples were incubated for 3 h at 39 °C, 5% CO_2 and 100% humidity in a high-cAMP medium (cocktail), already successfully demonstrated for capacitating ram spermatozoa. After pre-incubation with 4 or 40 μM mifepristone (dissolved in PBS with 0,1‰ DMSO), 1 μM P4 was added to samples. Two samples without P4 were included as controls, the cocktail-sample and another one incubated in TALP medium. Analyzed parameters were sperm motility (CASA), plasma membrane integrity (CFDA/PI stain), capacitation status by chlortetracycline (CTC) staining and tyrosine phosphorylation of membrane proteins (by western blotting), and phosphatidylserine (PS) translocation (Annexin V/PI stain) as an apoptotic marker. Data were compared by Chi-squared test. The results obtained showed that the presence of P4 in capacitating conditions led to a significantly higher percentage of acrosome-reacted spermatozoa compared with the sample without hormone (43.3±3.5% vs. 24.0±1.1). Pre-incubation with mifepristone significantly inhibited the increment in rate acrosome-reacted sperm rate (17.0±1.5 and 16.0±1.5 for 40 μM and 4 μM mifepristone, respectively) and also decreased the percentage of sperm with phosphatidylserine translocation (20.7±3.4, 24.7±2.9 vs. 35.7± 2.4 for 40 μM, 4 μM and without mifepristone, respectively). However, mifepristone did not affect the percentage of motile and with integral plasma membrane spermatozoa. In conclusion, this study showed that P4 induces the acrosome reaction and apoptotic changes in ram spermatozoa and that these actions are mediated by sperm progesterone receptors, given that mifepristone is able to decrease both the acrosome reaction and apoptotic markers.
Grants: CICYT AGL 2014-57863-R and DGA 2016-A26 FSE.

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Effect of 15-deoxy-$\Delta^{12,14}$-prostaglandin j2 (PGJ2) on sperm motility and binding to *in vitro* cultured oviductal epithelial cells (OEC). A preliminary study in porcine

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Keywords: spermatozoa, boar, prostaglandin J2.

The effect of 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (PGJ2) on its receptor, the sperm peroxisome proliferation-activated receptor gamma (PPARγ), has been linked to numerous biological activities, including boar sperm capacitation (Soriano-Úbeda, 2013, The 1st EPICONCEPT Annual Meeting, Antalya, Turkey). The aim of this work was to evaluate the effect of PGJ2 at 10 µM [Santoro, J Exp Biol, 216(6), 1085-1092, 2012] on sperm viability, motility and binding to *in vitro* cultured oviductal epithelial cells (OEC). Spermatozoa samples were obtained from 4 ejaculates of 2 to 3 years-old boars of proven fertile. OEC were obtained from oviducts of 6 to 7 month-old sows slaughtered at an abattoir and cultured in monolayer (López-Úbeda, Asian J Androl, 18, 1-18, 2016). For viability and motility analysis, spermatozoa were incubated in a capacitation medium (TALP) in presence (PGJ2) or absence (ø) of 10 µM of PGJ2 (D8440, Sigma-Aldrich®, Madrid, Spain). The viability was analyzed at 1 and 30 min of incubation using the eosin-nigrosin staining. The motility parameters were determined by computer-assisted sperm analysis (CASA) at 1, 5 and 30 min of incubation. The binding to OEC was evaluated after insemination with $1x10^5$ spermatozoa/ml stained with bisbenzimide and 30 min of co-culture in TALP in PGJ2 and ø groups. After co-culture, inseminated monolayers were fixed with paraformaldehyde and analysed under fluorescence microscopy at 10x determining the number of attached spermatozoa to OEC per square millimeter (spz/mm^2). Independent t-test ($p<0.05$) were performed and the results were expressed as mean ± SD for viability and motility parameters or SEM for binding to OEC. The viability of spermatozoa was not affected by the presence of PGJ2 both at 1 and 30 min of incubation. Several CASA parameters were influenced by PGJ2 presence throughout the incubation. At 1 min, PGJ2 produced a higher percentage of motile spermatozoa (%Mot: PGJ2: 93.9%; ø: 90.7%), progressive spermatozoa (%Prog: PGJ2: 46.6%; ø: 43.6%), curvilinear velocity (PGJ2: 146.3±89.6 µm/s; ø: 131.3±81.2 µm/s) and amplitude of lateral head displacement (ALH: PGJ2: 3.1±1.9 µm; ø: 2.8±1.6 µm). At 5 min, PGJ2 showed a higher linearity (LIN: PGJ2: 38.5±25.9%; ø: 32.7±24.0%) and wobble (WOB: PGJ2: 56.3±22.7%; ø: 53.8±21.8%), whereas at 30 min, none of the CASA parameters showed statistical differences between groups. When PGJ2 was added to OEC-spermatozoa co-culture, the number of bound spermatozoa was 27.5% lower than in ø (PGJ2: 85.2±3.4 spz/mm^2; ø: 117.5±3.6 spz/mm^2). Concluding, PGJ2 did not affect sperm viability, however it produced an immediate effect on motility, which probably increased the energy expenditure of the spermatozoa via activation of PPARγ, allowing a lower binding of spermatozoa to OEC. Supported by MINECO-FEDER AGL2012-40180-C03-01 and AGL2015-66341-R.

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NOS/NO modulate the protein phosphorylation on serine and threonine residues during boar sperm capacitation

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Keywords: nitric oxide, sperm, capacitation.

Spermatozoa need to undergo a series of functional changes before they can fertilize, which constitute the process known as capacitation. This involves the early activation of protein kinases and the inactivation of protein phosphatases [Battistone MA, Mol Hum Reprod. 19(9):570-80; 2013]. It has been reported that NO can be generated by spermatozoa during capacitation and it can modulate this process through protein S-nitrosylation [Lefièvre L, Proteomics. 7(17):3066-84; 2007], activation of the cAMP/PKA pathway [Belén Herrero M, Free Radic Biol Med. 29(6):522-36; 2000], but also by increasing the cGMP concentration [Murad F, Recent Prog Horm Res. 49:239-48; 1994; Wiesner B, J Cell Biol. 142(2):473-84; 1998]. High levels of cGMP may inhibit cAMP degradation, which leads in turn to PKA activation [Kurtz A, Proc Natl Acad Sci U S A. 95(8):4743-7; 1998]. The aim of this study was to further investigate the NO's involvement in PKA activation during the *in vitro* capacitation of boar spermatozoa. For this purpose, ejaculated sperm were incubated for 1 hour in capacitating and non-capacitating conditions (TALP medium [Rath D, J Anim Sci. 77(12):3346–52; 1999] and PBS (D1408, Sigma-Aldrich, St. Louis, USA), respectively). The media were supplemented with 100 µM S-Nitrosoglutathione (N4148, Sigma-Aldrich, St. Louis, USA), a NO donor, and two NOS inhibitors: 10 mM N^G-Nitro-L-arginine Methyl Ester Hydrochloride (483125, Merck, USA) and 10 mM Aminoguanidine hemisulfate salt (A7009, Sigma-Aldrich, St. Louis, USA). The pattern of protein phosphorylation on Ser and Thr residues was evaluated by Western blotting. The antibodies used in this study were: rabbit monoclonal antibody anti-protein kinase A (9624, Cell Signaling Technology, Beverly, USA, 1:2000) and goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz Biotechnology, USA, 1:10000). The relative amount of signal in each membrane was quantified using the ImageQuant TL v8.1 software (GE Healthcare, Life Sciences, Buckinghamshire, UK). Our results indicated that when capacitated in the presence of NOS inhibitors, spermatozoa showed a lower Ser and Thr phosphorylation pattern than those capacitated with or without the NO donor. This effect was not observed under non-capacitating conditions. In conclusion, this study provides additional evidence that NOS/NO plays a role in regulating the phosphorylation of Ser and Thr residues during sperm capacitation in porcine. Supported by European Union, Horizon 2020 Marie Sklodowska-Curie Action, REPBIOTECH 675526 and MINECO-FEDER (AGL 2015-66341-R).