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Transcript abundance and antioxidant biomarker of buck semen cryopreserved with melatonin supplementation

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Keywords*:* Keywords: bucks, melatonin, motility, total antioxidant capacity, gene expression.

This study was carried out to improve the freezability of buck semen using two different types of cryoprotectants with two doses of melatonin as antioxidant. Pooled samples from four sexually mature Egyptian Baladi Bucks were used in this experiment. Semen was diluted (1:8) of Tris-fructose-citric extender containing egg yolk. Either Glycerol or Dimethyl sulfoxide (DMSO) was used as cryoprotectant at a final concentration of 5 %. Extended semen was supplemented with different levels (10^{-6}mM and 10^{-9}mM) of melatonin (M5250, Sigma-Aldrich, St Louis, MO, USA) in addition to control group and cooled at 5 °C for 4h before deep-freezing at -196°C. Extended semen of all groups was adjusted to the same concentration and finally packed in 0.25 ml French straws (IMV).Computer assisted semen analysis (CASA) was used to evaluate semen after cryopreservation. Data was analyzed using the SAS GLM procedure (SAS, 2004) and applying the following model. Duncan's multiple range test was used to detect differences among means, the significance level was set at $P<0.05$. Quantitative real-time PCR data was analyzed using delta delta Ct method and values were reported as relative expression of target genes to the calibrator after normalization to reference gene (GAPDH). the progressive motility was higher ($p < 0.05$) in control sample extended with glycerol (71.6±2.3%) than that supplemented with DMSO (32.9±2.5%). The progressive motility was higher ($P<0.05$) in samples supplemented by low dose of melatonin (10^{-9} mM) compared with high dose (10^{-6}mM) in glycerol (74.4±2.4% and 64.4±2.5%, respectively) and in DMSO based extender (35.5 ±2.4% and 32.9 ±2.5%, respectively). The CASA parameters (VAP, VCL and VSL um/s) were significantly different in low melatonin dose from high melatonin dose in glycerol based extender being (57.4±1.1, 103.5±2.9 and 42.5±0.8) against (51.3±1.2, 91.8±3.0 and 37.7±0.8) respectively. The activity of total antioxidant capacity (TAC) was significantly greater in DMSO group supplemented with the low melatonin dose (0.49 mM/L ±0.09) than high melatonin dose (0.16 mM/L ±0.09) group. While, there was no significant differences in TAC between glycerol extender groups. Transcript abundance of genes enhancing mitochondrial activity CPT2, ATP5F1A and SOD2 was significantly ($p < 0.05$) increased in glycerol based extender groups and this was more apparent in low melatonin dose compared with all other glycerol based extender groups. On contrast, gene regulating oxidative stress (NFE2L2) was up-regulated ($p < 0.05$) in groups cryopreserved with DMSO extender compared with those cryopreserved in glycerol based extender. It could be concluded that using glycerol based extender supplemented with low concentration of melatonin would be recommend for enhancing the fertilizing ability of buck semen.

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Effects of increasing concentrations of LPS on *in vitro* ovine oocyte developmental competence

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Keywords*:* inflammation, lipopolysaccharide, oocyte developmental competence.

Negative energy balance (NEB) during early lactation in dairy cows leads to an altered metabolic state that has major effects on animal reproduction. Feeding high concentrate diet, a common strategy for mitigation of NEB, enhance the risk of ruminal acidosis. Both ruminal acidosis and infectious diseases can enhance the concentration of lipopolysaccharide (LPS), an important bacterial component in circulation leading to disturbed reproductive performance. Nevertheless, some degree of LPS induced inflammation be beneficial through triggering antioxidant process to protect cell from oxidative stress. Although considerable number of researches investigated the effects of LPS on reproductive performance of dairy cows, the response of sheep to the increasing concentrations of environmental LPS is not defined yet. Ewes ovary were collected from slaughterhouse, sliced and the oocytes with more than three layers of cumulus cell and integrated cytoplasm were matured for 24 h under increasing concentrations of LPS (0, 0.01, 0.1, 1 and 10 µg/mL). In order to measure the intracellular glutathione (GSH) content, a number of matured oocytes were denuded and stained with cell tracker blue and then observed using an epifluorescence microscope and were analyzed by ImageJ software. A number of matured oocytes also were fertilized using frozen ram semen. Then, the rate of oocytes reached to the blastocyst stage were recorded at day 8 post-insemination. Data were analyzed with GLM procedure of R software. Our data showed that there was no difference ($P \geq 0.05$) between the groups in GSH content, although it was higher in medium with 10 µg/mL of LPS. Addition of LPS reduced the number of fertilized oocytes reached to blastocyst stage in a dose dependent manner (36.69, 34.21, 35.41, 16.66 and 14.28 % of oocytes reached to blastocyst stage, respectively for 0, 0.01, 0.1, 1 and 10 µg/ml of LPS; $P<0.05$). It has been shown that LPS induces the production of pre-inflammatory cytokines such as (Interleukin 6) IL-6 and (Interleukin-8) IL-8 from variety of cells. In mammals, transcription factors such as (nuclear factor-κB) NF-κB and IFN are activated after recognition of LPS by Toll like receptor (TLR-4). Moreover, bovine granulosa cells express TLR4 receptor complex and response to LPS through phosphorylation of TLR signaling components p38 and extracellular signal-regulated kinase and increase the IL-6 and IL8 transcripts. LPS was reported to affect intracellular redox status and increase apoptosis through enhancing pro-apoptotic factors. A group of antioxidant enzymes and non-enzymatic processes protects gametes and embryos against ROS damage during oocyte maturation and early stage of development. In this study, although the difference between groups in regards to GSH content was not significant but maybe higher concentration of glutathione in response to high level of LPS was a mechanism for confronting the inflammatory response created in those groups. In conclusion, our results demonstrate that LPS in 1 and 10 µg/mL concentrations may have detrimental effects on oocyte developmental competence in ovine.

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The CatSper inhibitor effect on porcine sperm in the presence of higher chemotactic activity of the follicular fluid

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Keywords*:* Boar spermatozoa, NNC effect, follicular fluid.

Several components in follicular fluid (FF), in particular progesterone, have chemotactic capacity and depend on the entry of Ca2+ through membrane or CatSper channels in some species (Lishko P.V. et al., Nature, 471(7338):387–91 (2011)). On the other hand, P4 does not seem to be a clear CatSper agonist in porcine (Vicente-Carrillo A. et al., ReproBiol, 17 (1): 69-78 (2017)). The NNC 55-0396 inhibitory effect on CatSper in sperm has been demonstrated. The aim of this study was to investigate NNC effect in the presence or not of FF. The chemotaxis system used consists of two wells (A and B) connected by capillaries. Four wells (A) were filled with fresh sperm were first washed in a discontinuous gradient of Percoll®, followed by TALP medium (20x106/mL diluted in 500 µL) from proved fertility boars (N=4) previously incubated or not with NNC alone, NNC and 0.25% of FF, while the opposite wells (B) were filled with TALP (control group) and TALP supplemented with 0.25% of FF. NNC (2 µM) without cytotoxic effects and 0.25% of FF were used. Two experiments were performed: Experiment I: the A and opposite B were filled with 1: Control group (TALP (A) – TALP (B)), 2: FF+ (TALP (A) – FF (B)), 3: NNC (TALP+NNC (A) – TALP (B)), 4: FF- (TALP+NNC (A) – FF (B)). Experiment II: the A and opposite B were filled with 1: Control group (TALP (A) – TALP (B)), 2: FF+ (TALP (A) – FF (B)), 3: NNC (TALP+NNC (A) – TALP (B)), 4: NNC+FF (TALP+NNC+FF (A) – FF (B)). In experiment I, treatment 3 and 4 were preincubated (10 min) with NNC before chemotaxis, likewise, for treatment 3 in experiment II. However, the treatment 4 in experiment II was preincubated (10 min) at the same time with NNC and 0.25% of FF before chemotaxis. After 20 min of chemotaxis, the sperm concentrations (%) from wells B were evaluated using the free statistical software, Sas University Edition (SAS, 2016). In experiment I, the highest percentage of attracted sperm was seen in FF+ (9.1%b) versus control group TALP (7.5%a), NNC group (5.6%a), and FF-group (6.8%a) ($p<0.05$). Similarly to experiment II: FF+ (5.7%b) versus control group (4.6%a), NNC group (3%c), and NNC+FF group (3.9%a) ($p<0.05$). These results may indicate the chemotactic effect of FF on boar spermatozoa. Moreover, NNC inhibited sperm chemotaxis even in the presence of higher chemotactic activity of FF, which suggests that at least the chemoattractant components in FF might act via CatSper. Further studies should be carried out to test this hypothesis.

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Factors affecting the sperm concentration assessment in commercial seminal doses in pigs

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Keywords*:* sperm assessment, quality control, sperm concentration.

Artificial insemination is widely implemented worldwide with more than 90% of the sows inseminated with fresh semen. However, up to now, a quality control system for swine artificial insemination centers has not been internationally established. Evaluation of the sperm concentration in commercial seminal doses is a key point in the control of the dose's quality, since a clear relationship is established between total sperm number in the insemination dose and fertility outcome. The use of cell counting chambers is a cheap and simple methodology. Nevertheless, it tends to be less precise than other more sophisticated and expensive methodologies (Hansen, Theriogenology, 66, 2188. 2006). The aim of this study was the evaluation of different factors (dilution rate, pipetting repeatability, microscopy magnification, time of sample resting before evaluation and chamber area evaluated) that could modify the results in sperm concentration of commercial seminal doses, for further proposal of a scientific base a standardized protocol. Sperm concentration in 27 seminal doses was evaluated by one observer by dilution (1/10 or 1/20 rate) in saline solution (0.3% formaldehyde), pipetting by triplicate, disposed in a Neubauer chamber and observed after 1 or 5 minutes by contrast phase microscopy (x100, x200 or x400 magnification) and counting the number of spermatozoa present in 0.12 or 0.2 mm^2. Data were expressed as the mean ± SEM and analysed by ANOVA, considering the specific factors (dilution, pipetting, time, objective, area) as the main variable and sample as covariate. Bland-Altman analysis was applied to assess the degree of agreement, showing the bias (mean±SD). The pattern of relationship between difference and average was evaluated by lineal regression as quality of the agreement ($p<0.05$). Sperm concentration was not affected by dilution rate 1/10 or 1/20 (bias 1±5.42, p=0.74). No differences were found for concentration of samples by 3 pipetting procedures (p=0.81), between 1 or 5 minutes in the chamber before examination (p=0.73) and between counting areas of 0.12 vs. 0.20 mm^2 (p=0.69). However, the concentration measured using x10 objective was higher (41.56±2.54x10^6/ml) than using x20 (37.81±1.95, p=0.03) and x40 objectives (38.03±1.89, p=0.02). These differences were confirmed with significant regression for the difference x40-x10 (bias -3.53±9.14, p=0.01) and 20x-10x (bias -3.75±9.76, p=0.02). The overestimation with x10 objective could be related to difficulties to observe with precision the limits of the counting area or measuring as spermatozoa other different particles. These problems are minimized when higher magnification is used, although time consuming is higher with higher magnification. So, according to the obtained results, we propose a standard procedure with the selection of x20 contrast phase objective, dilution 1/20, 1 min sample resting and 0.2 mm^2 to optimize precision and time consuming.
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L-carnitine supplementation to UHT skimmed milk-based extender improves motility and membranes integrity of chilled ram sperm up to 96 h

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Keywords*:* L-carnitine, sperm, ram.

The addition of new additives as L-carnitine (LC) to extenders could mitigate reactive oxygen species (ROS) production and improved motility and viability in chilled ram sperm as has been demonstrated in other species (e.g. human, bull, and mice). The aim of this work was to evaluate the antioxidant effect of LC on motility variables and integrity of plasma, acrosomal, and mitochondrial membranes of chilled (5 ºC) ram sperm up to 96 h. Twelve pools from 36 semen ejaculates were collected by artificial vagina from 12 Merino rams (3-9 years) in four sessions during non-reproductive season (June to August). Each pool was divided into 6 aliquots and then diluted at $200x10^6$ sperm/ml in UHT-based extender (skimmed milk-6% egg yolk) supplemented either 1mM (LC1), 2.5mM (LC2.5), 5mM (LC5), 7.5mM (LC7.5), and 10mM (LC10) of LC. A control group without LC was included in each pool. Sperm motility variables were assessed by CASA system (SCA®) and total sperm with intact plasma membrane / intact acrosome / intact mitochondrial membrane (IPIAIM,%) was assessed by triple fluorescence association test (PI/PNA-FITC/Mitotracker green) at 0, 48, and 96 h. The effects of LC concentration and cold-storage time were analyzed by one-way ANOVA and Bonferroni´s test ($p<0.05$). Overall, the results showed that kinetic variables and integrity of sperm membranes decreased ($p<0.05$) as cold-storage time increased in all groups. The results revealed a higher ($p<0.01$) sperm motility (SM,%) in all LC groups than control group at 48 h. However, at 96 h, both LC5 and LC10 groups showed a SM higher ($p<0.001$) than both LC7.5 and control group (87.9±2.2 and 88.0±1.8 vs 82.9±2.1 and 82.5±3.1, respectively). Progressive sperm motility (PSM,%) was higher with LC5 group than control group at 48 h (42.2±2.9 vs 36.7±1.8) and 96 h (35.7±3.4 vs 29.0±1.7). Surprisingly, straight line velocity (VSL, µm/s) was improved with all LC groups compared with control group at 0h ($p<0.01$), 48h ($p<0.001$) and 96 h ($p<0.001$). Moreover, at 96h VSL (µm/s) value was higher with LC7.5 group than all LC groups ($p<0.5$) and control ($p<0.001$) (LC7.5: 87.2±4.9 vs LC1: 75.1±4.5, LC2.5: 78.6.2±5.9, LC5: 79.4±5.0, LC10: 79.3±5.2, and control: 65.4±3.4). Likewise, IPIAIM percentage was higher ($p<0.001$) in all LC groups than control group during at 48 h and 96 h (LC1: 62.3±2.0, LC2.5: 66.3±1.7, LC5: 63.3±2.8, LC7.5: 66.5±2.4, and LC10: 66.3±1.9 vs control group: 49.2±2.9). These results revealed a kinetic-enhancer effect of LC supplementation to UHT skimmed milk-based extender, which might improve fertility following cervical insemination of sheep.

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Identification and localization of NADPH oxidase 5 in ram spermatozoa

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Keywords*:* ram sperm, NADPH oxidase, melatonin.

The aim of this study was to identify the presence of NADPH oxidase 5 (NOX5) in ram spermatozoa and to investigate if melatonin could modulate this enzyme during *in vitro* capacitation. Semen from nine *Rasa Aragonesa* rams was collected and pooled. Seminal plasma free spermatozoa were selected by a swim-up procedure (control sample). Spermatozoa were then incubated in TALP medium without (TALP sample) or with cAMP-elevating agents (cocktail sample, Ck) for 3 h at 39 °C and 5% CO_2. 1 µM melatonin was added to TALP and cocktail samples (TALP-Mel and Ck-Mel) (n=6). Capacitation status was evaluated by chlortetracycline (CTC) staining. Identification and distribution of NOX5 in ram spermatozoa was investigated by western-blot and indirect immunofluorescence (IIF) with the anti- rabbit NOX5 C-terminal antibody (ab191010, Abcam, Cambridge, UK). At least 200 spermatozoa were scored per sample in CTC and IIF assays. Differences between experimental groups in CTC staining and NOX5 immunolabeling were compared by means of chi-square test using GraphPad InStat software (Version 3.01). As expected, the inclusion of cAMP-elevating agents in the cocktail sample increased the capacitated-sperm pattern by CTC compared with TALP sample after *in vitro* capacitation ($p< 0.001$), whereas the presence of melatonin at 1 µM in both samples increased the non-capacitated-pattern relative to samples without hormone ($p<0.001$). Regarding the presence of NOX5 in ram spermatozoa, Western blot analyses revealed a band of 86 kDa compatible with that reported to NOX5 in human (Musset et al., The journal of biological chemistry, 287: 9376-9383,2012) and equine (Sabeur and Ball, Reproduction 134:263-270, 2007) spermatozoa. IIF revealed six differences immunotypes depending on the presence of NOX5 in the ram sperm: I: apical region II: acrosome , III: post-acrosome, IV: apical and post-acrosomal, V: acrosome and post-acrosome (all subtypes with midpiece labelling) and VI: labelling in the midpiece of the spermatozoa. In swim-up selected (control) ram spermatozoa, the predominant NOX5 immunotypes were I and II. After incubation in capacitating conditions, these inmunotypes decreased in TALP samples and increased those III and V ($p< 0.001$) when compared to control. In cocktail samples, there was also an increase in the rate of spermatozoa with labelling only in the midpiece of the flagellum (type VI, $p<0.001$). However, the presence of melatonin in TALP medium (TALP-Mel) increased II subtype and in cocktail sample (Ck-Mel) increased V immnutype ($p< 0.001$),spermatozoa presented a NOX5 distribution very similar to that observed control and TALP samples respectively. In conclusion, these preliminary results reveal for the first time that NOX5 is present in ram spermatozoa, and that melatonin can prevent the NOX5 distribution changes associated with sperm capacitation.

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Effect of repeated ejaculates on seminal plasma composition and semen liquid storage in INRA180 ram

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Keywords*:* repeated ejaculate, semen storage, seminal plasma INRA 180 ram.

In sheep, superior rams are used extensively for mating or as sperm donors for genetic improvement. Nevertheless, sperm production capacity and its storage are a major factor confining extensive use of rams over numerous ewes for a long period of time. The objective of this experiment is to evaluate the effect of repeated ejaculates on liquid storage sperm motility, and seminal plasma biochemical composition in INRA180 ram. Five INRA180 rams were collected weekly for 4 weeks at a rate of three ejaculates each 20 minutes. Concentration of total protein (g / l) (Prot) (Lowry et al.,1951. *J. Biol. Chem. 193, 265-275*), total lipid (g / l) (Lip) (Woodman and Price, 1972. *Clin. Chim. Acta., 38, 39-43*) and fructose concentration (g / l) (Fruc) (Mann, 1948, *J. Agric. Sci., 38, 323-331*) were evaluated. Immediately after collection and initial evaluation, the semen was extended in skim milk (SM) at 15°C to reach 0.8×10^9 spermatozoa/ml. Thereafter, the samples were evaluated at different storage times (0, 8, and 24 h). A CASA system was used to determine total (TM%) and progressive (PM%) motilities. All analyses were carried out using a statistical software program JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA). Variance analysis (one-way ANOVA) was performed. The statistical model included fixed effects of repeated ejaculates (first, second and third). When statistically significant differences were detected, the Tukey's post hoc, was used to compare the means and standard errors for Prot, Lip and Fruc in seminal plasma, TM and PM in each storage duration (0, 8 and 24h) considering the significance level of $P < 0.05$. The results showed that, in seminal plasma, the total protein concentration was significantly higher in the first (25.30±0.22 g/l) and second ejaculates (25.17±0.29 g/l) compared to the third (24.36±0.23 g/l). The highest total lipid concentration was recorded in the first ejaculates (3.75±0.07 g/l) ($P <0.05$), followed by the second ejaculates (3.63±0.08 g/l) ($P <0.05$), while the third ones (3.46±0.09 g/l) recorded the lowest total lipid concentrations ($P <0.05$). The fructose concentration was higher in the second ejaculates (5.49±0.16 g/l) ($P <0.05$), followed by the first ejaculates (5.39±0.14 g/l) ($P <0.05$), while the third ejaculates recorded the lowest fructose concentrations (4.96±0.17 g/l) ($P <0.05$). Regarding semen liquid storage, the results indicated that the second ejaculate has significantly better sperm motility compared to the first and the third ones and this still true until 8 h of liquid storage. While at 24 h, the first ejaculate gives the best results ($P <0.05$). To conclude, our results recommend the use of the second ejaculate for artificial insemination before 8 h of storage and the first ejaculate until 24 h of storage.

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Effects of semen collection methods and equilibration times on post-thaw sperm kinematic parameters of Saanen bucks

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Keywords*:* electro-ejaculation, equilibration time, sperm kinematics.

The success of AI depends on semen quality and female fertility. Buck semen is commonly collected by artificial vagina (AV). However, when bucks cannot be trained for semen collection or semen is collected to evaluate fertility of bucks before mating season, the electro-ejaculation (EE) method is usually utilized. However, differences on sperm characteristics between ejaculates collected by AV and EE have been reported. The equilibration process and type of extender are known to affect the quality of post-thaw sperm quality and this study hypothesize that semen collection methods also have confounding effects. The present study was conducted to investigate the effects of semen collection methods and equilibration times on sperm kinematic parameters of Saanen bucks. Eight bucks were divided into two equal groups (4 bucks) based on semen collection methods; AV or EE. A total of 12 ejaculates (collections) per buck were collected at weekly intervals. Freshly collected ejaculates were pooled per group and extended with clarified egg-yolk tris extender (CEY). Pooled semen samples were cooled to 4^{o}C within 2 h and equilibrated at 4^{o}C for 2, 4 and 6 h in separate aliquots. Thereafter, the samples were then frozen using standard procedure after completion of each equilibration time. Four straws of frozen semen per group per collection were thawed at 33°C for 30 sec and evaluated for post-thaw sperm motility and kinematic parameters using CASA system 24 h after freezing. Semen collected with AV had significantly ($p<0.001$) higher sperm curvilinear velocity (VCL: 122.21±1.23 μm/s), straight line velocity (VSL: 89.24±0.11 μm/s), linearity (LIN: 64.23±0.91 %), beat/cross-frequency (BCF: 7.21±0.02 Hz), total motility (98.12±0.34 %), rapid sperm (66.26±0.11 %) and progressive motility (77.51±1.12 %) compared to semen collected with EE method. Post-thaw sperm curvilinear velocity (VCL: 65.52±0.02 μm/s) were higher ($p<0.001$) for sperm equilibrated for 2 h in semen collected with AV methods compared to other equilibration times. Straight line velocity (VSL: 49.15±0.92 μm/s) was higher ($p<0.001$) for sperm equilibrated for 2 h in semen collected with AV methods. Average path velocity (VAP: 64.65±0.43 μm/s) was higher ($p<0.001$) for sperm equilibrated for 4 h in semen collected with AV. Linearity coefficient (LIN: 74.34±1.01 %) and straightness coefficient (STR: 77.89±0.45 %) in semen collected with AV were higher ($p<0.001$) for sperm equilibrated for 2 h compared to semen collected with EE and other equilibration times (4 and 6 h). Amplitude of lateral head displacement (ALH: 3.46±0.98; 3.39±0.02 μm) was higher ($p<0.001$) for sperm equilibrated for 4 h in semen collected with AV and 2 h equilibration in semen collected with EE. The AV method and equilibration times for 2 or 4 hours preserved sperm motility and kinematic parameters post-thaw.

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Description of porcine spermatozoa-interacting proteins after contact with male and female reproductive fluids

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Keywords*:* porcine, biological fluids, sperm proteome.

After ejaculation, sperm are deposited within the female genital tract by natural or artificial insemination, starting their journey towards the fertilization site. Along the way, sperm take contact with reproductive fluids, and only few of them reach the oocyte. The first fluid in which sperm are immersed during ejaculation is seminal plasma (SP), involved in aiding sperm transport and survival through the female genital tract by SP-proteins, improving their fertilizing ability (Bromfield, Animal, 104-109, 2018). Once deposited within the uterus, sperm contact with uterine fluid (UF), a dynamic female milieu that changes its proteome during the oestrus cycle (Soleilhavoup, Mol Cell Proteomics, 93-108, 2016). In sow, sperm-UF interaction is still unknown, but UF exerts a cytotoxic effect on sperm cells unprovided of SP (Kawano, Proc Natl Acad Sci, 4145-50, 2014). After crossing the uterus, selected sperm reach the oviduct, interacting with the oviductal fluid (OF), inducing sperm functional changes (Perez-Cerezales, Biol Reprod, 262-276, 2018). Since sperm interaction with fluids may change sperm proteome, the aim of this study was to identify proteins that adhere to ejaculated sperm (S) after contact with different reproductive fluids [SP, UF and OF (collected in slaughtered sows)] for a better understanding of sperm behavior during their journey previous to fertilization. The experimental groups used were: 1) S group (control): sperm without reproductive fluids; 2) SP group: S incubated with 20% SP; 3) UF group: S incubated with 20% UF (late follicular phase); 4) OF group: S with 20% OF (late follicular phase); 5) UF-SP group: S with 20% UF and 20% SP; 6) OF-SP group: S with 20% OF and 20% SP. All the groups were incubated for 180 min at 38°C, then centrifuged at 600 *g* for 5 min and the pellet was used for protein extraction, carefully performed to detect surface proteins. Sperm proteome was assessed by HPLC-MS/MS analysis. The total number of proteins identified was 88. Among these, 56 proteins were detected in S group and in UF, SP and UF-SP groups. 72 proteins were detected in S group and in OF, SP and OF-SP groups. This study has also allowed to identify a higher number of proteins in common between OF and OF-SP groups (29), than in UF group and UF-SP group (3 proteins in common). Furthermore, sperm incubated with UF-SP showed a lower number of proteins (17) than when incubated with SP (32) or UF (42). Instead, sperm showed 44 proteins when incubated with OF-SP, 32 proteins with SP and 42 proteins with OF. One of detected proteins, sperm acrosome membrane-associated protein 1, was expressed in all the groups except in OF-SP group. Moreover, sperm equatorial segment protein 1 was detected in all the groups except in UF-SP group. The combined use of SP with UF or OF suggests an interaction between these fluids that modify the sperm proteins probably caused by a steric hindrance. In conclusion, this study highlights how sperm proteome changes after interaction with different reproductive fluids, with a potential physiological impact during the *in vivo* fertilization process. Supported by MINECO and FEDER (AGL2015-66341-R and AGL2015-70159-P) and Fundación Séneca (19357/PI/14).

A120E Physiology of reproduction in the male and semen technology

Nuclear morphometrics and chromosome positioning in boar sperm

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Keywords*:* sperm, nucleus, morphometrics.

The predicted increase in the global population and changes in dietary preferences have led to a rise in the demand for meat products. Artificial insemination is routinely used in commercial pig breeding, for which the use of high-quality semen samples during insemination is crucial. With an aim to reduce inter-operator variability and the laborious nature of manual semen analysis, we have developed a fast, unbiased software-based approach which allows comprehensive analysis of pig sperm nuclear morphometrics. Fresh ejaculated semen samples were identified as either fertile or sub-fertile using a combination of computer assisted sperm analysis (CASA) and manual assessment prior to use of samples in this study. Using CASA, 'normal morphology' was assigned to samples if less than 30% of the sample contained morphological defects such as bent tails, coiled tails, distal midpiece reflex (DMR), proximal droplets and distal droplets. Subjective manual assessment was used to score motility from 1 to 5, 1 being dead and 5 being excellent. Samples were categorised as fertile if more than 70% of the sample had 'normal morphology' and if at least 85% of the sample had a motility score of 4 or above. Those falling below these criteria were categorised as sub-fertile. Analysis of nuclear morphology from 50 fertile and 50 sub-fertile samples yielded measures from 11,534 and 11,326 nuclei respectively. Cluster analysis using measures of Area, Circularity, Variability, Bounding height and Bounding width by Ward linkage using squared Euclidean distance and standardised variables supported the existence of three clusters with different membership for fertile and sub-fertile sperm. Specifically, sperm heads from fertile animals were overrepresented in one cluster and underrepresented in another. The cluster in which sperm heads from fertile samples were overrepresented was characterised by a high mean nuclear area, which was a consequence of greater head width, and by low variability between sperm heads. We extended this analysis to determine if chromosome positioning in pig sperm also varies between fertile and sub-fertile samples. In a preliminary study, two fertile and two sub-fertile semen samples from Pietrain boars were analysed using fluorescence *in situ* hybridisation with locus-specific subtelomeric probes, and the position of pig chromosomes 10 and X were determined. This suggested that chromosomal position also differs between nuclei from fertile and sub-fertile samples. Based on this preliminary finding, we are currently extending this study to perform a complete analysis of nuclear organisation using a larger sample size of 20 samples and imaging more cells per sample. In conclusion, we show that there are morphological and chromosome positioning differences between sperm nuclei from fertile and sub-fertile samples. This approach therefore has the potential both to be used as a tool for sperm morphology assessment and as a way to investigate the causes of fertility differences.

A121E Physiology of reproduction in the male and semen technology

Season affects refrigerated-stored semen doses from a commercial stud AI centre: A flow cytometry study of sperm physiology and chromatin status

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Keywords*:* spermatozoa, pig, seasonality.

The pig industry rely on the production of semen doses in stud centers, which are distributed to the production farms. Advances in boar selection, extender formulation and storage allow for consistency on sperm quality and artificial insemination (AI) results. However, seasonality still affects semen quality (Porcine Health Manag 3:15, 2017). Our objective was to characterize the influence of the season in the doses produced in a modern center (NE Spain). We tested two hypotheses: Sperm quality was affected by the season, and this effect followed a yearly sinusoid pattern. Semen doses (40×10^6 ml^{-1}) were produced from 236 Pietrain boars in routine semen production (extender from Magapor, Zaragoza, Spain). The doses (436 from early 2017 to early 2019) were sent at 17 °C to the laboratory, being analysed by 48 h of storage. An aliquot was added to the staining solution at 10^6 ml^{-1} (BTS with Hoechst 33342, PNA-FITC, merocyanine 540, propidium iodide and Mitotracker deep red; ThermoFisher, Waltham, MA), for viability, acrosomal status, capacitation, and mitochondrial activity assessment (Theriogenology 80, 400–410, 2013). Another aliquot was submitted to ORT (osmotic resistance test, 15 min in 150 mOsm/kg BTS before staining). After 15 min at 37 °C, samples were run in a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotech, Bergisch Gladbach, Germany). Sperm chromatin was assessed by SCSA (Sperm Chromatin Structure Assay; Methods Cell Sci 22:169–189, 2000), obtaining %DFI (DNA fragmentación) and %HDS (chromatin immaturity). Data were analyzed with the R statistical package, testing season effects with linear mixed-effect models (calendar season as fixed and male as grouping factors) and cosinor regression. We also tested the relationship between physiological and chromatin variables by Pearson correlations. Doses collected were 111 in spring, 96 in summer, 117 in fall and 112 in winter. Sperm quality was overall good (interquartile ranges, viability: 79.6, 89.6; intact acrosomes: 88.4, 93.2; viable-capacitated: 2.9, 5.5; viable-active mitochodria: 71.1, 97.2; ORT: 61.6, 87.1; %DFI: 0.4, 0.7; %HDS: 0.5, 1.6). We detected a season effect in viability, acrosomal integrity, mitochondrial activity, and %HDS ($P<0.001$) , and in viable capacitated ($P=0.003$). Cosinor detected a yearly sinusoid pattern ($P<0.025$) (peak and low-point indicated) for: viability and mitochondrial activity (mid-spring/mid-fall); acrosomal integrity (early-fall/early-spring); capacitated (late fall/late spring); and %HDS (early summer/early winter). We also found significant correlations of %HDS with acrosomal integrity (-0.66, $P<0.001$) and mitochondrial activity (0.40, $P=0.048$). Overall, the effect size of the calendar season was small. However, its influence on the %HDS, being a chromatin structure parameter, merits study. Stud centers should take these results into account, since the season effect may increase in suboptimal situations, affecting the adherence to quality standards. Supported by RTI2018-095183-B-I00 (Ministry of Science, Innovation and Universities, Spain) and AGL2016-81890-REDT (MINECO, Spain). We thank EVB (Spain) and Lucía Tejerina for their collaboration in this study.

A122E Physiology of reproduction in the male and semen technology

SLO1 channels are essential for acrosome reaction during *in vitro* capacitation of boar spermatozoa

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Keywords*:* sperm; capacitation; acrosome reaction; pig; SLO1 channels.

The aim of the present study was to determine whether SLO1 channels, also known as big potassium (BK) or maxi K^+ channels, are involved in sperm capacitation and acrosome reaction in boar spermatozoa. With this purpose, we incubated semen samples from five boars in *in vitro* capacitation medium plus paxilline 100 nM (PAX), a specific blocker of SLO1 channels. Sperm samples were incubated in capacitation medium at 5% CO_2 and 38.5ºC for 240 min, with or without PAX. At 240 min, progesterone was added to control and PAX samples to induce the acrosome reaction. Samples were incubated for further 60 min (300 min). After 0, 60, 120, 180, 240, 250, 270 and 300 min of incubation, total and progressive motility were measured by Computer Assisted Sperm Analysis (CASA), and acrosome integrity, permeability of plasma membrane, and intracellular calcium levels measured by Fluo3 and Rho5 were determined by flow cytometry. After confirming that data distributed normality and variances were homogenous, a mixed model followed by post-hoc Sidak test was run. Total and progressive motility, as well as calcium levels measured by Fluo3, which preferentially stains calcium residing in the mid-piece, did not differ significantly between control and PAX samples at any incubation time ($P>0.05$). Despite permeability of plasma membrane and acrosome integrity being lower in PAX than in control samples after the addition of progesterone, these differences were not significant ($P>0.05$). In contrast, intracellular calcium levels measured by Rhod5, which has more affinity for calcium residing in the sperm head, and acrosome reacted spermatozoa were significantly ($P<0.05$) lower in PAX than in control samples after 250 and 300 min of incubation. We can thus conclude that, while SLO1 channels do not seem to play a key role for motility regulation of boar spermatozoa during capacitation and progesterone-induced acrosome exocytosis, they are essential for triggering the acrosome reaction. This involvement appears to be related with the modulation of calcium stores present in the sperm head.

A123E Physiology of reproduction in the male and semen technology

In vitro assessment of sperm characteristics using semen from Norwegian Red bulls with high and low fertility

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Keywords*:* bull, non-return rate, sperm characteristics.

The aim of this study was to investigate possible associations between *in vitro* parameters and fertility by assessment of several *in vitro* sperm characteristics and IVF using cryopreserved semen from Norwegian Red bulls of contrasting fertility. The bulls were characterized as low- or high-performing bulls based on non-return rate after 56 days (NR56) for an average of 1132 first AIs per bull, ranging from 47% to 79%. NR56 was calculated as LSmean for 507 bulls used in AI from 2013-2018, based on a General Linear Model (PROC GLM in SAS®) including the following parameters: bull, AI month and year, parity and double AI within 1-4 days. Totally 37 bulls with contrasting NR56 were selected for analyses, 19 bulls with NR56 LSmean ranging from 0.76 to 0.78 and 18 bulls ranging from 0.46 to 0.65. Cryopreserved semen doses were analysed for total sperm motility, progressive motility and hyperactivity by computer-assisted sperm analysis. Additionally, the ATP content was assessed using the CellTiter-Glo® Luminescence assay. Sperm chromatin, acrosome and plasma membrane integrity were analysed by flow cytometry using the Sperm Chromatin Structure Assay, Alexa 488 conjugated peanut agglutinin and propidium iodide, respectively. Furthermore, semen from selected bulls from the contrasting fertility groups were used for IVF. *In vitro* production of embryos were performed with media from IVF Bioscience using four well plates containing 500 µL of the respective media. Bovine ovaries were collected at a local slaughterhouse, transported to the laboratory and cumulus–oocyte complexes (COCs) were aspirated from follicles sized 3 to 15 mm in diameter. Groups of high quality COCs were matured for 22 h (6% CO_2, 38.8°C). Spermatozoa prepared at a concentration of 1×10^6 /ml were added to each group of oocytes followed by 18 h incubation (6% CO_2, 38.8°C). Cumulus cells were removed from the presumptive zygotes by vortexing prior to cultivation in a humidified atmosphere (7% O_2, 6% CO_2 and 87% N_2). At day 3 post-fertilization, the cleavage rate was evaluated. Further, the blastocyst rate at day 7 and day 8 was recorded. Cryopreserved semen from each bull was used in three replicate experiments including 180 oocytes per bull. Statistical analyses were performed by linear mixed models in Rstudio (v 1.1.463) using the *in vitro* sperm parameters and LSmean for NR56 as dependent and independent variables, respectively. In addition, bull, age and season at the time of semen collection were included in the model. Total motility, progressive motility and hyperactivity was positively associated with NR56 ($p<0.05$). Furthermore, sperm chromatin integrity, calculated as DNA fragmentation index and high DNA-stainable sperm, showed a negative association with NR56 ($p<0.05$). The ATP content and acrosome integrity were not associated with NR56. Preliminary results from IVF indicate that bulls of similar fertility (NR56) obtain different blastocyst yields *in vitro*. In conclusion, the results of the study showed that NR56 was associated with several *in vitro* sperm parameters. Prediction of fertility might be possible combining *in vitro* sperm analyses, where the use of IVF could provide valuable additional information.