Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

Sexually inexperienced photo-stimulated bucks increased plasma testosterone concentrations during first contact with nulliparous and multiparous anestrous goats

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Keywords: goats, naïve male goat, photoperiod

In sheep, social isolation from ewes decreases testosterone secretion in males. Furthermore, sexually inexperienced rams secrete low concentrations of testosterone during their first interaction with ewes. This study aimed to determine whether sexually inexperienced photo-stimulated male goats increase plasma testosterone secretion during first contact with nulliparous and multiparous anestrous females. This study was performed in Torreon, State of Coahuila, Mexico (25°23' N, 104°47' W; 1200 m above sea level). Male kids (*n*=6) were born in December and were weaned at 40 days of age. Male goats were housed together in a pen and were totally isolated from any visual, auditory, tactile, and olfactory sensory signal from female goats. At 10 months of age males were subjected to a photoperiodic treatment of artificially long days from November 1 to January 15 (16 h of light and 8 h of darkness per day), from this date onward males perceived the natural photoperiod until the end of the experiment. Nulliparous (*n* = 30) and multiparous (*n* = 30) females were used. Before introduction of the males with females a transrectal ultrasonography was performed to determine the ovulatory state using an Aloka SSD-500 equipped with a 7.5 MHz transducer. An anovulatory state was established in those females in whom a corpora lutea were not detected. All females were anovulatory. In males, blood samples to determine plasma testosterone concentrations were collected by venipuncture of the jugular vein. Blood samples were taken weekly when the photoperiodic treatment ended from January 16 until May when the study ended. Plasma testosterone concentrations were determined using ELISA. In march, when the males were 15 months of age were randomly separated into two groups of 3 each, and were exposed for the first time to nulliparous and multiparous females (1 male × 10 females) for 15 consecutive days. Plasma testosterone concentrations were analyzed using a completely randomized model with repeated measures using generalized estimation equations. Plasma testosterone concentrations changed over time between the two groups of males; interaction week × group of males. P<0.001). Photo-stimulated males increased plasma testosterone concentrations in the first week of March during first contact with nulliparous (10.8 ± 2.6 ng/mL vs first week of January, 1=2.0 ± 0.7; P<0.05) and for multiparous females (9.3 ± 1.9 ng/mL vs first week of January, 1=1.16 ± 0.14; P<0.05). In conclusion, sexually inexperienced photo-stimulated males increased plasma testosterone concentrations during first contact with nulliparous and multiparous anestrous females.

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PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

Bicarbonate and caffeine trigger capacitation-like changes (acrosome damage) in boar sperm after 2 h in vitro incubation.

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Keywords: in vitro capacitation, sperm, porcine, BSA, bicarbonate, caffeine

Sperm capacitation is a crucial process within the reproductive female tract. This complex mechanism is associated with biochemical changes that enable spermatozoa to bind, penetrate and fertilize the egg. Hyperpolarisation of the plasma membrane, change of ions concentration levels (including calcium uptake), phosphorylation of sperm proteins, ROS production or changes in motility patterns are involved in sperm capacitation. These modifications rely, at least in part, on different compounds that we could find in the female genital tract, such as bicarbonate. The purpose of this study was to assess, *in vitro*, the effect of the inclusion of different molecules (bicarbonate, bovine serum albumin (BSA), and caffeine) on sperm quality parameters after 2 h incubation at 38.5 °C. To address these effects, we used eighteen boar commercial artificial insemination doses to test, in e*xperiment 1*, three concentrations of bicarbonate (19, 37 and 56 mM; n=6), in *experiment 2,* three concentrations of BSA (1.5, 3 and 4.5 mg/mL; n=6) in a based media (BM) supplemented with 37 mM of bicarbonate. Finally, in *experiment 3, the* absence or presence of caffeine (5.15 mM; n=6) in the BM containing bicarbonate (37 mM) and BSA (3 mg/ml) was analysed. The BM contained sodium chloride (NaCl: 112 mM), potassium chloride (KCl: 4.02 mM), sodium phosphate monobasic monohydrate (Na2HPO4: 0.83 mM), magnesium chloride (MgCl2: 0.52 mM), D-(+)-glucose (13.9 mM), sodium pyruvate (1.25 mM), and calcium chloride (CaCl2: 2.25 mM; 0.25 mg/mL). Total motility (TM), membrane integrity (VIAB) and acrosome damage (ACR; H33342/PI/PNA), capacitation status (chlortetracycline staining CTC), and mitochondrial membrane potential (JC1) were analysed. Experiment 1 revealed that higher concentrations of bicarbonate (37 and 56 mM) decreased (P<0.01) TM and VIAB but increased (P<0.01) ACR after 2 h of incubation compare to the fresh control. In contrast, in experiment 2, the BSA concentration of 3 mg/mL reduced only the VIAB, whereas all the concentrations tested increased the average membrane potential (JC1) and decreased TM after incubation in comparison to the fresh control. Finally, in experiment 3, the capacitated pattern measured by the CTC technique and ACR increased after 2h of incubation compared to fresh control, although no differences were found in the presence/absence of caffeine. Our results showed that, according to our *in vitro* experimental conditions, bicarbonate is the main compound triggering capacitation-like changes in the boar sperm. This effect could be explained, at least in part by the already described presence of a bicarbonate gradient in the female genital tract that may be linked to the release of sperm from the female sperm reservoirs. Further studies are needed in order to elucidate the specific mechanism by which the boar sperm is capacitated *in vitro*.

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PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

Evaluation of the progesterone inhibitor RU486 in boar sperm cryopreservation

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Keywords: sperm, porcine, RU486, cryopreservation, mifepristone

Boar sperm cryopreservation is a sub-optimal method due to the high sensitivity of sperm to cold shock. In addition, sperm cryopreservation induces capacitation-like changes, such as sperm membrane modifications, thereby affecting calcium channels, which causes variations in the ion exchange. These structural changes and intracellular signalling lead to increased acrosomal reactions or different patterns of movement, called cryo-capacitation. On the other hand, progesterone (P4) is an inductor of capacitation, including acrosomal reaction, mainly by inducing changes in the plasmatic membrane that allow the entry of calcium, which in turn activates the signalling pathway of adenylate cyclase and the production of cAMP. Being said that the use of a specific progesterone inhibitor could revert the P4 action, this preventing capacitation-like changes that happen during sperm cryopreservation. The aim of this study was to evaluate the effect of different concentrations of a progesterone inhibitor (RU486) in the freezing extender, on post-thaw boar sperm quality. Throughout the experiment, commercial artificial insemination semen doses from six fertile boars were centrifuged and diluted (300 x 10^6 sperm/mL) in a freezing extender (LEY: 20% egg yolk and 80% lactose), supplemented with RU486 (A: non-supplemented (control); B: 5 mM; C: 10 mM; D: 20 mM). The samples were cooled to 5 °C, extended (1:3; v:v) in LEYGO (LEY + 9% glycerol and 1.5% Equex) and 0.5 mL straws frozen in LN_2 vapours, and thawed at 37 °C/20 sec. Total motile sperm (%TMS) and other kinetics parameters were evaluated with the IA Station CASA system (SPERMTECH®). Furthermore, membrane integrity and acrosome damage were determined by using triple staining (Hoechst 33342/propidium iodide PI/PNA-FITC) under epifluorescence microscopy. Sperm with undamaged membrane and acrosome (%VIAB) or reacted acrosome (%VIAB/AR) and sperm with damaged membrane and acrosome (%DEAD/AR) were recorded. Moreover, sperm with high membrane potential (%HMP) were analyzed by using double staining (Hoechst 33342/JC1). Data were analyzed using SAS (Version 8.2, SAS Institute Inc., Cary, NC, USA). The GLM procedure evaluated the effects of the freezing extender on the different sperm quality parameters. Results are shown as the mean ± standard deviation, these outcomes from the addition of different concentrations of RU486 failed to improve (P<0.05), relative to the control group, any of the parameters evaluated post-thawing. These results expressed in %TMS (A: 31.7 ± 18.0, B: 33 ± 18.3, C: 35.9 ±3.4, D: 24.8 ± 14.3), %VIAB (A: 76.4 ± 10.9, B: 69.03 ± 12.8, C: 67.9 ± 6.6, D: 66.23 ± 9.3), %VIAB/AR (A: 0, B: 0, C: 0, D: 0.12 ± 0.3), %DEAD/AR (A: 3.6 ± 1.9, B: 6.05 ± 4.9, C: 6 ± 3.3, D: 4.9 ± 3.1) and %SHMP (A: 29.93 ± 33.9, B: 24.18 ± 19.4, C: 23.3 ± 21.7, D: 23.7 ± 32). In conclusion, the progesterone inhibitor RU486 does not affect any post-thawing sperm quality parameter analyzed. Further studies are needed to test whether the combined use of RU486 and progesterone could induce a reduction in cryo-capacitation changes.

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PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

Bioenergetics changes during in vitro capacitation of ram spermatozoa

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Keywords: sperm capacitation, energy metabolism, hyperactivation

Several biochemical and physical changes take place during sperm capacitation to prepare spermatozoa for oocytes' fertilization. Such changes, especially those related to modifications in the motility pattern (hyperactivation) and the increment of tyrosine phosphorylation, seem to require more energy. In spermatozoa, ATP is the primary source of energy and is mainly produced through oxidative phosphorylation (OXPHOS) and glycolysis. However, the metabolic exigencies during capacitation, including the preferred metabolic pathway, seem species-specific, and little is known about it in ram spermatozoa. The present study investigated metabolic variations during *in vitro* capacitation in ram spermatozoa together with diverse kinematic parameters to better understand this event. After removing the seminal plasma from ejaculates (n=9) with a density gradient (Bovipure®), ram spermatozoa were incubated at 38.5°C from 0 to 180 min under capacitating (CAP) and non-capacitating conditions (NC). The CAP medium was composed of synthetic oviductal fluid (SOF) with 10% of estrous sheep serum, while the NC medium by SOF with 0.1% polyvinyl alcohol. The ATP content and lactate excretion rate (final subproduct of glycolysis) were evaluated using a luminometer. The percentage of capacitated and acrosome-reacted sperm were assessed with a fluorescence microscope using the chlortetracycline fluorescence assay (CTC). Kinematic parameters associated with the acquisition of hyperactivation (curvilinear velocity: VCL; linearity: LIN; and amplitude of the lateral displacement: ALH) were measured by the CASA system. After 180 min, the proportion of capacitated and acrosome-reacted sperm (AR) increased ($p<0.05$) under CAP conditions compared to NC conditions (65.34 ± 2.18% vs. 13.50 ± 2.67% CAP; 28.16 ± 3.22% vs. 16.40 ± 2.75% AR). VCL and ALH increased ($p<0.05$) at the same time that LIN decreased ($p<0.05$) in those sperm incubated under CAP conditions at 180 min compared to NC conditions (VCL: 144.89 ± 4.26µm/s vs. 55.01 ± 4.78 µm/s; ALH: 3.35 ± 0.08 µm vs. 1.19 ± 0.1µm; LIN: 29.02 ± 2.93% vs. 48.12 ± 3.45%), which confirms the presence of the hyperactivated-like pattern. The ATP content of sperm was drastically reduced ($p<0.05$) after 180 min under CAP conditions in comparison to NC conditions (60.63 ± 4.21amol vs. 111.36 ± 3.86amol). Finally, the production of lactate also decreased ($p<0.05$) after 180 min under CAP conditions compared to NC conditions (6.19 ± 2.34nmol vs. 9.91 ± 2.53nmol). Our results revealed that *in vitro* capacitation of ram spermatozoa increases ATP consumption, which seems to promote a shift in the usage ratio of the two metabolic pathways mentioned, from glycolysis to OXPHOS. In addition, the increment of ATP demands during sperm capacitation could be in part attributed to the noted increase in the hyperactivated-like population.

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PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

Multidimensional characterization of the pig sperm chromatin by flow cytometry

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Keywords: Boar, semen, chromatin, DNA fragmentation

Artificial insemination (AI) is critical in the modern pig industry. The status of the sperm chromatin is often overlooked but is responsible for many cases of boar subfertility (low prolificacy and economic losses). Classical parameters analyzed, such as DNA fragmentation, only consider one aspect of the complexity of sperm chromatin structure. This study analyzed by flow cytometry 181 AI doses (22 boars, 2 collections, 3 ejaculates/week/collection) from a stud center in León (Spain) after production (day 0, D0) and after 11 days (D11) of storage at 17 °C. The analyzes were the DNA fragmentation index (SD-DFI and %DFI) and chromatin maturity (%HDS) by SCSA (Sperm Chromatin Structure Assay); Disulfide bridges between protamines (disulfide index [DB], median fluorescence intensity [MFI] and cell% of high, moderate and low thiol presence) by monobromobimane (mBBr) staining; Chromatin compaction (MFI and cell% for high, intermediate and low compaction) by chromomycin A3 (CMA3); and DNA oxidative damage by 8-oxo-dG immunostaining. The data were analyzed by Pearson correlations (false discovery rate adjustment), variable clustering (Hoeffding D statistic), and principal component analysis (PCA) with hierarchical clustering in the multidimensional space defined by the parameters and the relationship with the sources of variation (male, collection, and storage). Parameters within the same technique showed moderate to high correlations and significant ones among techniques, especially after storage. Thus, these techniques could identify different and potentially complementary characteristics in the boar sperm chromatin. At D0, 8-oxo-dG positively correlated with SCSA variables and mBBr MFI and negatively with the DB, whereas %HDS positively correlated with %high-mBBr, mBBr MFI, and low CMA3 and negatively with %moderate-mBBr and the DB. At D11, 8-oxo-dG correlated with SD-DFI and %HDS, and %HDS with %high-mBBr. The variable clustering was more similar before and after the storage, tending to associate the mBBr parameters with the SCSA ones and 8-oxo-dG at D0 and clustering out the CMA3 parameters. At D11, the mBBr MFI and the DB clustered with the CMA3 parameters, suggesting a modification in the chromatin structure possibly stemming from the reorganization of the disulfide bridges. The PCA resulted in a first principal component (PC1) influenced mainly by mBBr parameters and a second one (PC2) by SCSA and the 8-oxo-dG. The hierarchical clustering showed the storage day as the factor contributing the most to the variability among observations and along the PC1. PC2 did not result in cluster separation but suggested that boars mostly differed according to SCSA and 8-oxo-dG. This study demonstrates the potentiality of simultaneously using different chromatin analyses for boar sperm characterization and multidimensional techniques to interpret the data emerging from these analyses. Further studies should test if the variability due to the storage and the male explain fertility differences after AI.

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PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

A new protocol combining uterine fluid and sperm selection improves porcine in vitro fertilization output

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Keywords: biofluids, fertility, pig, selection.

From the ejaculation to the fertilization place, the sperm cross a dynamic environment within the female reproductive tract taking contact with different biofluids proceeding from male (seminal plasma, SP) and female (uterine fluid, UF; oviductal fluid). These fluids contribute to sperm selection so that the most suitable sperm can reach the oocyte. Thus, our aim was to improve knowledge about the effect of SP and/or UF on the sperm fertilizing ability before and after an in vitro selection, mimicking the in vivo events. For this purpose, we performed the in vitro fertilization (IVF) after contact with SP and/or UF before and after an in vitro selection by density gradients. Ejaculates were collected from 4 boars with proven fertility, and centrifuged (500 g, 5 min) to remove the SP. Four experimental groups were prepared ($20x10^6$ sperm/ml): 1) Control, sperm without biofluids (in PBS); 2) SP, sperm with 20% SP; 3) UF, sperm with 20% UF; 4) SP+UF, sperm with 20% SP and 20% UF. The samples were incubated for 3h at 38°C, then split in two aliquots, one of which was centrifuged (700 g, 5 min) with a capacitation medium, the other one was in vitro selected by Percoll density gradient 45/90% (700 g, 30 min). Then, each group was used to perform the IVF, and the following parameters were evaluated: penetration rate (%), monospermy rate (%), and efficiency (% of monospermic oocytes from total number of penetrated oocytes). A normality test followed by ANOVA test was performed by SAS (2016). When the IVF was performed before the selection, penetration rate was significantly higher in control (57.52±11.36%) than SP (21.08±7.64%), UF (24.20±5.26%), and SP+UF groups (17.40±8.30%) ($p < 0.001$), without differences in monospermy. Then, the efficiency was calculated, resulting in a higher percentage in control (45.60±4.93%) than SP (17.05±4.84%), UF (18.87±2.10%), and SP+UF groups (14.15±6.02%) ($p < 0.001$). Interestingly, after in vitro selection, sperm previously incubated with UF showed a penetration rate significantly higher (UF= 69.88±4.05%; SP+UF= 66.66±6.58%) than control (46.10±8.36%) and SP (48.12±11.86%) ($p < 0.02$), without difference in monospermy. Regarding the efficiency, UF and SP+UF groups were significantly higher (53.43±2.80%, 51.24±3.88%, respectively) than control (39.18±5.71%) and SP groups (31.74±4.64%) ($p = 0.01$). Based on the results obtained, UF incubation combined with a sperm selection induces a marked improvement in IVF. Therefore, further studies are needed to find possible biomarkers in the new combined sperm preparation system (UF and sperm selection) here presented responsible for this improvement.

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PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

The reproductive fluids surrounding boar sperm before in vitro selection determines sperm molecular changes

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Keywords: biofluids, molecular markers, reproduction

The events prior to fertilization are characterized by the sperm interaction with different molecules proceeding from the surrounding environment. While crossing the male and female tracts sperm metabolize several substrates from the fluids in which they are immersed (seminal plasma–SP, uterine fluid–UF) and adsorb lipids from them inducing changes in sperm membrane composition (Shan, Int J Mol Sci; 22(16): 8767, 2021; Menezes, BMC Genomics; 20(1):714, 2019). These components may contribute to the selection of sperm suitable for fertilization. Thus, we investigated sperm lipidome after incubation with SP and/or UF and in vitro sperm selected by density gradient. Ejaculates were collected from 5 boars with proven fertility, and centrifuged (500 g, 5 min) to remove the SP. Four experimental groups were prepared ($20x10^6$ sperm/ml): 1) Control, sperm without biofluids (in PBS); 2) SP, sperm with 20% SP; 3) UF, sperm with 20% UF; 4) SP+UF, sperm with 20% SP and 20% UF. The samples were incubated for 3h at 38°C, then in vitro selected by Percoll density gradient 45/90% (700 g, 30 min) was performed. Each sample was centrifuged at 5200 g for 10 min at 4°C and stored at -80°C until analysis. Then, lipidome profiling was assessed by UltraPerformance Liquid Chromatography coupled to HighResolution Mass Spectrometry. Once the data were obtained, they were processed by LipidMS R-package, grouped by lipid species, and subjected to a univariate T-test analysis. Sperm lipidome results revealed a higher amount of total lipids in UF than Control, SP and SP+UF groups (p=0.01). In particular, glycerophosphoserines, glycerophosphoethinolamines, triglycerides, fatty acids, monoglycerides, diglycerides, ceramides and sphingosine were more abundant in UF than the other groups (p<0.01). Monoglycerides were also more abundant in SP than control and SP+UF groups (p<0.04). Regarding glycerophosphoethinolamines, fatty acids and monoglycerides, they were also more abundant in SP than SP+UF (p=0.04). In conclusion, the difference in sperm lipidome profiles depends on the fluid with which they take contact. In particular, the presence of UF increases the sperm lipid concentration, suggesting its involvement in the improvement of the reproductive performance.

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PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

Validation of the open source OpenCASA sperm motility analysis software in three wild ruminant species

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Keywords: wild ruminants, semen, motility, CASA, methods agreement

Computer-assisted sperm analysis (CASA) is a technology enabling the assessment of sperm quality by automatic or semi-automatic evaluation of microscopic images or videos, typically for morphology (CASA-morph) or motility (CASA-mot) assessment. Currently, CASA includes analyses of features such as viability or DNA damage. Whereas the development of CASA has been parallel to proprietary software, open-source solutions have been proposed. OpenCASA was presented recently and has been tested in several species (Alquézar-Baeta et al. PLoS Comput. Biol. 15:e1006691, 2019). In this study, OpenCASA v. 2 was validated with ISAS v. 1.019 CASA software (Proiser, Valencia, Spain) for analyzing the motility of several wild ruminant spermatozoa: Red deer (n = 32), roe deer (n = 15), and chamois (n = 36). Image sequences were acquired from epididymal samples on a Makler chamber at 37 °C with a Nikon E600 microscope (10× negative contrast) and a Basler A312fc camera at 53 images/s, 25 image/s for roe deer samples. Total and progressive motility and kinematic parameters were obtained for each sample in both softwares (semi-automatically in ISAS and automatically in OpenCASA), using the same parameters for defining sperm detection (head area and motility features). The concordance correlation coefficient (CCC) and the Bland-Altman method (bias and coefficient of agreement) estimated between-method agreement (R statistical environment). CCC (rho with 95%CI) showed a high or moderately high agreement for total and progressive motility (roe deer: 0.79 and 0.89; red deer: 0.95 and 0.87; chamois: 0.90 and 0.69). Bias (mean ISAS-OpenCASA difference) and coefficient of agreement for total motility were 9.4 and 10.5 for roe deer, -3.0 and 11.7, and 0.35 and 7.4; and for progressive motility were -1.5 and 13.7, -6.5 and 9.9, and -13.2 and 9.4. Kinematic parameters (velocities and linearity estimations) showed CCC rho values around 0.7 and, in general, bias and coefficients of agreement below 10. These results indicate a good agreement between the two methods for these species; differences between methods could be explained by the algorithmic determination of sperm tracks, which are undisclosed in proprietary software. Since OpenCASA is based on open-source software, it could be easily expanded and adapted for other lab needs and analysis systems.

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PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

The sperm DNA oxidative damage and fragmentation are early predictive factors of the in vivo fertility of boar

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Keywords: boar, semen, chromatin, DNA oxidation, DNA fragmentation, fertility

Artificial insemination (AI) is the modern industry's standardized pig production system. Therefore, early detection of subfertile boars is essential to minimize economic losses in farming. Subfertility has been linked to DNA instability and fragmentation, and it usually goes unnoticed in field conditions. We hypothesized that DNA oxidative damage (a determinant for DNA fragmentation) could be an early predictive factor of boar subfertility. This work adapted the 8-hydroxy-2'-deoxyguanosine (8OHdG, a marker of global DNA oxidative damage) immunodetection to study boar spermatozoa and its relationship with DNA fragmentation and boar fertility. AI doses from 18 boars (3 ejaculates/boar, 54) were donated by an insemination center near León (Spain). Fertility data was provided for the boars (parity and prolificacy, results after AI in productive conditions), classifying them in high (H), medium (M), and low (L) in vivo fertility. The AI doses were analyzed by flow cytometry right after production (day 0, D0) and after 11 days of storage at 17 °C (D11). The parameters used in the study were: mean fluorescence intensity (8OHdGMFI) and % of positive spermatozoa (%8-OHdG+) after 8OHdG immunodetection, as measurements of DNA oxidative damage; and DNA fragmentation as %DFI (spermatozoa with increased DNA fragmentation index) and chromatin maturity as %HDS (spermatozoa with elevated DNA stainability) from the Sperm Chromatin Structure Assay (SCSA). The data were analyzed by linear mixed-effect models (effect of the fertility group) and Pearson's correlations (association among chromatin parameters). Group L showed a higher %DFI than M at D0 ($p<0.05$) and higher %HDS, 8OHdGMFI, and %8-OHdG+ than M and H both at D0 ($p<0.05$) and D11 ($p<0.001$). The L boars also showed a significantly higher %8OHdG+ at D11 than at D0 ($p<0.001$). The association analysis indicated that %8OHdG+ strongly correlated with 8OHdGMFI ($r=0.941$, $p<0.001$), and moderately with %HDS ($r=0.688$, $p<0.001$). 8OHdGMFI showed a moderate correlation with %HDS ($r=0.673$, $p<0.001$). This study suggests that the sperm DNA oxidation status could indicate boars' potential fertility. 8OHdG immunodetection could be used at the beginning of the productive life of young boars to optimize the selection criteria of the best individuals for production maximization. Specifically, DNA oxidation status and chromatin immaturity could be valuable for the discrimination of subfertile males. Thus, the classical SCSA and the novel 8OHdG immunodetection techniques should be considered for integrated use in a new generation of advanced tests in pig farms. Further studies should test the relation of DNA oxidation on other productive parameters and the extrapolation to other mammal species.

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THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

Effect of date palm pollen on ram semen quality parameters during liquid storage at 5°C

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Keywords: ram semen, liquid storage, phoenix dactylifera L. pollen

The *Phoenix dactylifera* L. date palm pollen (DPP) is widely used in the traditional pharmacopeia of Moroccan oasis population. In fact, pure or mixed to medicinal plants, pollen powder is used to stimulate lactation in humans (Baliga et al., 2011; Hassan, 2011) and cattle. DPP and male palm flowers were traditionally claimed to be aphrodisiacs and fertility enhancers. In this context, the present study aimed to determine the phenolic compounds profile of the methanolic (MeOH) extract of DPP and its effects on Sardi ram semen quality parameters during liquid storage at 5 °C for up to 24 hours in skim milk extender (SM). To achieve this, DPP extracts were obtained after maceration in solvents of increasing polarity (hexane, methanol, ethanol, ethyl acetate and water) overnight in the dark at 30°C before undergoing evaporation under low pressure (Rotary evaporator Buchi R-210). The extracts were then tested for their radical scavenging abilities. MeOH, showing the best results, was chosen to continue this study and was analyzed for its phenolic compounds profile using high performance liquid chromatography coupled with a diode array detector (Shimadzu Cooperation, Kyoto, Japan) (Tokul Ölmez et al., 2020). Otherwise, semen samples were collected from six rams and extended with SM supplemented with DPP MeOH extract at 0, 1, 2 and 3 µg/ml to a final concentration of 0.8×10^9 spz/ml before being stored for up to 24 hours at 5 °C. The semen motility parameters were evaluated after 0, 4, 8 and 24 h using a computer-assisted sperm motility analysis (CASA; ISAS, version 1.0.17, Proiser, Valencia, Spain), while viability, abnormality and lipid peroxidation were evaluated using nigrosine eosin, Diff–Quik staining (Automatic Diagnostic Systems S.L., Spain) and measurement of thiobarbituric acid reactive species (TBARS) formed (Allai et al., 2016). SM supplemented with 3 µg/ml showed the best results and was then used to further detail this study. Statistical analysis was performed using JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA) program. The data, including motility, viability, abnormality, and lipid peroxidation, were tested for normality and homogeneity using the Shapiro–Wilk and Kolmogorov–Smirnov tests, respectively. A single-factor analysis of variance (ANOVA) was conducted to analyze the effects of the extracts at each storage time and the effect of storage duration in each extract. The results showed the presence of nine compounds belonging to the classes of phenolic acids and flavonoids in the pollen sample mainly ellagic acid, rutin and fisetin. Besides, a significant increase ($P < 0.05$) was noted in sperm total and progressive motilities, velocity and viability parameters in semen conserved with MeOH DPP extract added to skim milk compared to the control. Other beneficial effects have been recorded such as a significant decrease ($P < 0.05$) in sperm abnormality and lipid peroxidation. In conclusion, skim milk supplemented with 3 µg/ml of MeOH DPP extract enhanced ram semen quality after liquid storage. Further studies are required to verify these ameliorative effects on fertility rate in Sardi ewes during artificial insemination leading towards its incorporation into sperm extenders.

Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

Comparative study of Windsnyer and Large White boars sperm cryo survival rate

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Keywords: Windsnyer boars, Large White boars, cryopreservation

Cryopreservation allows preservation of genetic variability through biotechnological reproduction programs. However, approximately 40 to 60% of boar sperm do not survive cryopreservation process. The objective of this study was to compare sperm cryo survival rate of Windsnyer and Large White boars. Total of 36 ejaculates (6 replications/boar) were collected from three Windsnyer and Large White boars of proven fertility with the use of hand-gloved technique method, twice per week. Boars semen were pooled and extended with Beltsville Thawing Solution [(BTS) IMV Technologies, France], held at 18°C for 2 hours and centrifuged. The sperm pellet was re-suspended with Fraction A (20% egg yolk + BTS) and cooled at 5°C for 1 hour then diluted with Fraction B [3% Glycerol (Laboratory Consumables & Chemicals Supplies cc, Johannesburg, South Africa) + 20% egg yolk + BTS] and loaded into 0.25 mL straws (Embryo Plus, Brits, South Africa). The semen straws were placed on liquid nitrogen (LN_2) vapour for 20 minutes and then transferred to the LN_2 tank. Thawing was accomplished by immersing the semen straws in water at 40°C for 30 seconds. Sperm motility, viability and morphology characteristics were evaluated following thawing. Sperm motility was evaluated with the use of Sperm Class Analyser® (Microptin, Spain) system. Sperm viability and morphology were evaluated at 100X magnification under the microscope (Olympus, BX 51FT, Tokyo, Japan). The data were analyzed using the analysis of variance (general linear model) and statistical analysis system (SAS®). Treatment means were separated using Fisher's protected t-test the significant differences were determined by P-value at a significant level of P<0.05. Greater than 90% sperm total motility was recorded in the fresh semen of Large White and Windsnyer boars (P>0.05). Furthermore, highest frozen-thawed sperm total motility (51.1±12.7) and progressive motility (27.1±10.8) percentage was recorded in the semen of Large White boars (P<0.05). However, highest post-thawed live normal (31.8±6.7) and dead (67.8±7.2) sperm percentage was recorded in the semen of Windsnyer boars (P<0.05). Therefore, sperm cryo survival rate was maintained better in the semen of Large White boars.

Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

The potential of Spirulina platensis as a feed supplement for rams reared in endemic fluorosis areas to enhance semen characteristics and seminal plasma composition

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Keywords: Fluorosis, ram semen, Spirulina platensis

Fluorosis is caused by long-term ingestion of high levels of fluoride and has negative effects on sheep production. Moroccan sheep reared in endemic fluorosis areas are particularly susceptible to this condition which leads to decreased bodyweight, wool production, and fertility. Rams may also experience decreased testosterone levels, lower semen quality, and increased oxidative stress (Rahim et al., Toxicology 465, 153025, 2022). Chronic fluorosis is common in areas of Morocco where livestock production is a vital economic contributor. Thus, there is a need to address the effects of fluorosis on ram reproduction in these areas. The present study aimed to investigate the potential of *Spirulina platensis* as a feed supplement for rams reared in endemic fluorosis areas to enhance semen characteristics and seminal plasma composition. Twenty-one rams aged 5 months were chosen for the present experiment, and they were separated into 4 homogeneous groups on body weight (BW) basis, age, and initial health conditions. Among these groups, G0 (3 rams) and G1 (4 rams) served as controls belonging respectively to fluorosis free (Settat) and endemic fluorosis (El Fokra) areas. The other two groups, G2 (6 rams) and G3 (8 rams) (belonging to El Fokra) were respectively supplemented with 250 mg/kg BW and 500 mg/kg BW. All animals were allowed to be taken to pasture and reared extensively. The experiment was carried out for 13 months (from 5 to 18 months of age). During the last 4 months of the experiment, semen collections (n= 168) and measurements were performed every 15 days. Seminal plasma was tested for total proteins, lipid peroxidation, superoxide dismutase, reduced glutathione, catalase, and vitamin C. Statistical analysis was performed using JMP.SAS.VR 2011 software, including single-factor ANOVA and Dunnett test with a p-value of 0.05. The study showed a significant decline in semen characteristics in G1 compared to G0. However, when *Spirulina platensis* was added as a dietary supplement, there was a noticeable improvement in semen characteristics for the other two groups (G2 and G3). These improvements included an increased semen volume, a better mass motility, and fewer abnormalities. Additionally, these findings indicate that the levels of total proteins, vitamin C, and the antioxidant system in the seminal plasma of fluorotic rams were adversely affected. However, the use of *Spirulina platensis* as a dietary supplement restored these imbalances. In conclusion, this study highlights the negative impact of fluoride on ram semen characteristics and seminal plasma composition. It demonstrates the beneficial effects of *Spirulina platensis* as a dietary supplement as it enhances semen characteristics and balances the antioxidant system in fluorotic rams.

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THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

IZUMO1 is required for gamete fusion in rabbits

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Keywords: Fertilization, gamete fusion, knock-out, rabbit, CRISPR.

Fertilization requires the fusion of the sperm membrane with the oolema. Such cell fusion event is poorly understood, although a discrete number of proteins have been proved essential for this process by gene ablation experiments (knock-out, KO) in mice. Between them, IZUMO1 was the first sperm protein required for murine fertilization. However, it remains unclear whether IZUMO1 or other of the proteins deemed as essential based on mouse models are also required for fertilization in non-rodent mammals, as only TMEM95 has been proven to be required for fertilization in mice and cattle. The objective of this study has been to determine if IZUMO1, a protein essential for gamete fusion in mice, is also required for fertilization in rabbits, a phylogenetically distant species (*Lagomorpha*). To that aim, we have generated a line of *IZUMO1* KO rabbits by CRISPR technology. A mosaic male carrying wild-type (WT) and edited alleles was crossed with WT females. Within the heterozygous (Hz) offspring a KO allele composed by a deletion of 5 nucleotides at the beginning of the coding region was selected to establish the line (F1 generation). WT and Hz males (F2 generation) were able to father litters, but 9 females mated with 3 *IZUMO1* KO males (3 females/male) did not deliver. As expected, given the sperm exclusive expression of *IZUMO1*, female fertility was unaffected by the ablation. To identify a potential fertilization failure in sperm lacking IZUMO1, oocytes were collected from WT females mated with WT or KO males (3 females/group) at 15 hours post-insemination (hpi) by oviduct flushing. Cumulus cells were almost completely absent in both groups and presumptive zygotes were cultured up to 40 hpi to assess cleavage rate. All oocytes recovered from females mated by *IZUMO1* KO males (n=34) failed to cleave, whereas normal fertilization rates were observed for WT males (27/33). Following fixation and DAPI staining, no pronuclei was detected in the uncleaved oocytes recovered from the cross with KO males, and KO sperm were present their periviteline space, evidencing that sperm lacking IZUMO1 were able to reach the oocyte and traverse cumulus cells and zona pellucida, but were unable to fuse its membrane with that of the oocyte. In conclusion, the essential role of *IZUMO1* in gamete fusion is conserved between mice and rabbits.

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Animal Reproduction

Impact of ejaculate fractions used during artificial insemination on porcine uterine vascularization

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Keywords: ejaculate, uterine vascularization, pig

The interaction of the components of an ejaculate with the uterus may result in the modification of the uterine environment, including vascular changes of the endometrium that may influence fertilization (O'Leary, S. et al. *Reproduction* 1470–1626, 2004; Álvarez-Rodríguez, M. et al. *Int. J. Mol. Sci*. 21, 5477. 2020; Bogacki, M. et al. *Genes* 11, 1302. 2020). Then, we aimed to study the effect of accumulative ejaculate fractions in semen doses on the vascularity of different uterine regions in the sows. A total of 20 sows were divided into a non-inseminated group (control-C, n=5); and 3 groups of sows inseminated (AI group) with different semen fractions (F): F1, inseminated only with the rich fraction of the ejaculate (n=5); F2, inseminated with F1 + intermediate fraction (n=5); and F3, inseminated with F2 + poor fraction (n=5). At day 6 post-insemination, the sows were sacrificed and the uterus were collected. The 3 uterine areas evaluated were the following: Region 1, which belongs to the uterine horn close to the oviduct; Region 2, which corresponds to the central zone of the uterine horn; and Region 3, the uterine horn next to the uterine body. The uterine samples were routinely fixed and processed for paraffin embedding. The immunohistochemistry with the primary antibody CD31+ was performed to identify the vascular endothelium and the slides were digitized at 0.172 pixel/µm (Pannoramic MIDI II scanner3D Histech®). The entire field was photographed at 10x with the SlideViewer® microscope and the images (5-7 images/slide) were analyzed with ImageJ® to obtain a tissue segmentation to calculate the percentage of the vascular area. Statistical analysis (SPSS®) included an ANOVA, Pearson's R correlation and non-parametric Kruskal-Wallis test comparing C and AI groups, semen fractions (F1 vs. F2 vs. F3) and uterine regions (Region 1 vs. 2 vs. 3). Significant differences were considered when *p-value* <0.05. A total of 302 histological images were analyzed, which corresponds to 8.93 mm^2 of uterine tissue. The interaction of the ejaculate with the uterus represented a significant increase of 5.1 percent in the vascular area of the AI group compared to the C. All ejaculate fractions showed significantly greater percentage of vascular area compared to C, being F2 the one with the highest values (F1: 24.2 percent ± 1.15; F2: 27.5 percent ± 1.05; F3: 23 percent ± 1.03 vs C 19.8 percent ± 1.2, p<0.05). Focusing on each anatomical area, the F1 and F2 showed significantly higher percentage of vascular area than F3 in the region 1. However, no statistical differences were found between fractions in the regions 2 and 3. The percentage of vascular area in different areas of the endometrium of pregnant sows 6 days after AI is modified by the semen fraction of the ejaculate used to prepare the semen doses.

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Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

Localization of toll-like receptor 7 (TLR7) in bull, ram and dog spermatozoa and assessment of its use as a sexing target

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Keywords: sexing, spermatozoa, TLR7

The X-linked toll-like receptor 7 (TLR7) seems to be a useful target for immunological sex sorting of spermatozoa in bull, mouse and goat using a TLR7/8 ligand known as Resiquimod (R848) (Umehara *et al*., *Nature Protocols,* 15, 2645–67, 2020). Identification of a reliable target which can be used to sex spermatozoa would have great potential in both animal breeding and biomedical research on sex-linked diseases. Our objective was to localize TLR7 expression in the spermatozoa of bull, ram and dog and analyze its efficacy in separation of X- and Y-chromosome bearing spermatozoa.

The localization of TLR7 was determined via immunofluorescence staining of spermatozoa fixed on slides with 50:50 acetone:methanol (n=4 replicates). Slides were blocked for 1 hour with 5% normal goat serum in PBS and antibodies were diluted in this blocking solution [anti-TLR7; 1/100; BS-6601R, Bioss Antibodies, Woburn, USA][CY3 conjugated anti-rabbit; 1/400; A10520; Invitrogen, Waltham, USA]. TLR7 expression was categorized into negative or positive. Bull spermatozoa were incubated in a swim-up column of synthetic oviductal fluid (SOFaaci; Marei, Theriogenology, 86, 940-948, 2016) supplemented with 500 µM creatine, 2 mM glucose and 0.4% fatty acid free BSA +/- 0.03 µM R848 [HY-13740; MedChemExpress, South Brunswick Township, USA] following the method for bull sexing described by Umehara *et al*. Contrary to previous reports, TLR7 was found on the equatorial region of the sperm head and/or the tail region in bull sperm. Dog spermatozoa showed the same equatorial band with expression also on the post-acrosomal region of the sperm head; ram spermatozoa showed TLR7 expression homogenously throughout the entire post-acrosomal region of the sperm head. In assorted samples, approximately half (49% [SEM: 9%]) of bull spermatozoa expressed TLR7 indicating that TLR7 expression is restricted to X chromosome-bearing spermatozoa. After sexing, there was no significant change in the sperm concentration in the R848 treated sperm relative to control in either the bottom fraction of medium (7% [13%]) or the top fraction of medium (15% [24%]). After treatment with R848 only 24% [12%] of spermatozoa in the top fraction of medium expressed TLR7, suggesting that R848 treatment may have been effective, although this did not reach statistical significance via a paired T-test (p= 0.1552). No difference was observed in the proportion of TLR7 positive sperm in the bottom fraction of medium or in any of the controls. In contrast to bull spermatozoa, preliminary data indicated that 90% of dog spermatozoa expressed TLR7 (n=1). Similarly, 97% of ram spermatozoa expressed TLR7 (n=1), suggesting that R848 may not be an effective sexing target in these species, although further work is required to validate this. In summary, TLR7 expression appears to be localized to the spermatozoa head in dog, bull and ram, and is also expressed in the tail region of some sperm in bulls. Further work is required to determine the efficacy of utilizing R848 to separate X- and Y chromosome-bearing spermatozoa.

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

Sperm interaction with oviductal tissue in porcine

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Keywords: biomedicine, decellularization, sperm quality

Decellularization is a process by which cells and nuclear components are removed from a tissue in order to obtain decellularized extracellular matrix scaffolds to be used in a wide range of biomedical applications. One of the methods used for tissue decellularization is through detergents, which are toxic to cells, so they must be eliminated (Afarin Neishabouri, Frontiers in Bioengineering and Biotechnology, 10, 2022). Nevertheless, the effect the sperm interaction with reproductive decellularized tissues remains partially unknown.

Thus, the aim of this research was to study the effect of decellularized oviductal tissue (DOT) on porcine sperm quality over time. For this purpose, 4 experimental groups were prepared: 1) Control: semen sample (SS); 2) UW: SS + unwashed DOT; 3) W24: SS + DOT washed 24 h; 4) W48: SS + DOT washed 48 h. The oviducts were obtained from prepuberal sows, were dissected, and subjected to a decellularization process using the detergents Sodium Dodecyl Sulfate (SDS) (0.1%) and Triton X-100 (1%) (Sigma-Aldrich®, St. Louis, USA) under conditions of agitation at 150 rpm and refrigeration at 4 °C. For the W24 and W48 groups, the oviducts were washed for an additional 24 and 48 h respectively with Phosphate Buffer Solution (PBS) (Sigma-Aldrich®, Madrid, Spain). On the other hand, semen samples (n = 5) were obtained by gloved hand method from boars (initial criteria: motility ≥ 70% and morpho-anomalies ≤ 25%) and were diluted with ND-10 (IMV Technologies, L'Aigle, France) to obtain 30 × 10^6 spermatozoa/mL. Once prepared, the seminal sample was coincubated with 2 × 2 mm portions of the DOT according to the experimental groups and then, sperm quality was evaluated (motility and kinetic parameters were analysed by CASA system (Proiser R + D, Paterna, Spain) and sperm viability, acrosome integrity and mitochondrial activity by fluorescence microscopy (Leica® DM4000 Led, Wetzlar, Germany) immediately after preparation and after an incubation for 1 and 3 h at 38 °C. These parameters were compared by a sphericity for repeated measures test and was carried out using SAS University Edition program (SAS, 2016).The results showed that UW group negatively influenced sperm quality, significantly affecting total (71.07 ± 6.01) and progressive motility (35.47 ± 4.41) and viability (70.73 ± 3.33) parameters compared to the control (84,40 ± 2.26; 46.00 ± 3.47; 80.73 ± 2.40, respectively), with p < 0.01 in all cases. On the other hand, W48 group had no effect (p > 0.05) on viability (79,80 ± 3.08) and damaged acrosome (3.80 ± 1.09) compared to the control (70.73 ± 3.33; 3.07 ± 1.06), whereas the W24 group significantly (p < 0.05) affected viability (75.87 ± 2.20) and damaged acrosome (4.60 ± 0.97). In conclusion, W48 group had similar sperm quality to the control, hence, additional washes were required to completely remove residual detergents in the DOT and therefore avoid cytotoxicity and maintain sperm quality. In this way, interaction studies of reproductive decellularized tissue with sperm or other types of cells can be carried out.

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Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

Identification and quantification of tyrosine phosphorylated proteins induced by FERT medium during rabbit sperm capacitation at different times

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Keywords: rabbit spermatozoa, capacitation and tyrosine phosphorylated proteins

Rabbit sperm capacitation is still a largely unknown process. Thus, the main objetive of this study was to examine the molecular mechanisms that modulate capacitation in this species at different times. Semen from six New Zealand White x California male adult rabbits was collected free from gel and sediments and pooled. Spermatozoa was selected by a swim-up procedure: sample was centrifuged twice at 1000 g for 5 minutes at room temperature and incubated in 2 mL of Tyrode's medium in a tube 45° inclined for 20 min with 5% CO_2 and at 38.5 °C. The top layer (800 µl) was then incubated in FERT medium (Tyrode´s medium with 2 mM sodium bicarbonate, 36 mM sodium lactate, 1 mM sodium pyruvate, 0.2% fatty acid-free BSA, 10 µg/mL heparin and 0.001% (w/v) of phenol red) (Sigma; St. Louis, USA) for 4, 6 or 8 h at same conditions. Capacitation status was evaluated by chlortetracycline (CTC) staining. Identification and distribution of tyrosine phosphorylated proteins (TyrPP) in rabbit spermatozoa was investigated by indirect immunofluorescence (IIF) and western-blot (WB) using the monoclonal anti-phosphotyrosine antibody (4G10, Millipore; Massachusetts, EEUU) and anti-alpha tubulin as loading control in WB (mAB 926-42213; LICOR, Nebraska, EEUU). At least 200 spermatozoa were scored per sample in CTC and IIF assays (n=5). Five different immunotypes were stablished depending on Tyr-PP localization: I) non staining, II) equatorial region, III) equatorial and acrosome region, IV) flagellum, V) equatorial region or/and acrosome and flagellum of the spermatozoa. Differences between experimental groups in CTC staining and TyrPP immunolabeling were compared by means of X^2 test and the quantification of TyrPP-WB by image J analysis were statistically analysed by ANOVA using GraphPad InStat software. Incubation of rabbit sperm selected by swim-up in FERT medium during 8 h increased the percentage of capacitated (51.4 vs. 19.6%) and reacted (17.9 vs. 7%) sperm patterns by CTC compared with swim-up sample ($p< 0.001$). The localization of Tyr-PP in rabbit sperm by IIF revealed that in swim-up sample the immunotype III increased ($p< 0.001$) in relation to ejaculated sample. After incubation in *in vitro* capacitating conditions, the inmunotypes with labelling in the flagellum, IV and V increased ($p< 0.001$) compared to swim-up sample as in other species (Gimeno-Martos,Anim Reprod Sci.,221:106567,2020; Ruiz-Díaz, *Animals (10)*1467,2020) and could be related to changes in motility to promote oocyte binding. Also, WB analyses revealed an increase in the amount of TyrPP proteins (in the 37 KDa band) of capacitated samples relative to raw and swim-up samples compatible with results in rabbit sperm incubated with other medium during 16h (Saez-Lancelotti;*PLOS ONE*,5(10),e13457,2010). In conclusion, these preliminary results show, for the first time, that capacitation of rabbit spermatozoa can be induced by incubation in FERT medium for 8 h as demonstrated by increased percentage of Tyr-PP localization in the flagellum of spermatozoa and increased TyrPP levels.

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Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

Effect of different temperature conditions on sperm quality during storage: a comparison between sperm rich fraction and the whole ejaculate in boars.

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Keywords: storage, porcine, sperm quality

Lately, the application of semiautomatic sperm collection, which involves collecting the entire ejaculate, increased due to its practicality. This stands in contrast with traditional collection method, where only the sperm-rich fraction is used. Also, there has been a shift towards centralizing the distribution of semen doses in the swine industry, resulting in changes in the temperature conditions to which spermatozoa are exposed to during transport. Hence, the objective of the study was to assess sperm quality from the rich fraction and the complete ejaculate in different temperature conditions during storage. A total of 12 ejaculates (including 5 different boars of Pietrain breed) were used to form experimental groups: SR=spermrich fraction and BE=including the bulk ejaculate. Seminal doses (diluted in AndroStar® Plus extender; Minitüb, Tiefenbach, Germany) of each group (SR and BE) were kept in four temperature conditions: A) 15°C for 5 days (15-group); B) 5°C for 5 days (5-group); C) 5°C until day 3 then 15°C until day 5 (5/15-group); D) 15°C until day 3 then 5°C until day 5 (15/5-group). 5 ml of each group was stored with a concentration of $30x10^6$ sperm cells per ml. All samples were evaluated on day 1, 3 and 5 for motility and motion parameters by Computer Assisted Sperm Analysis (ISAS® software, PROiSER R+D S.L., Valencia, Spain), as well as for viability (propidium iodide), acrosome integrity (FITC-PNA) and mitochondrial membrane potential (JC-1) by fluorescence microscopy. On day 5 of storage, thermal- and cold-resistance tests were performed keeping aliquots in 38°C for 5 hours or in 0°C for 5 min, correspondingly. After that, the same parameters as mentioned above were evaluated. Statistical analysis was done by SAS OnDemand for Academics (2016) software using repeated measures design. To evaluate statistical differences after thermal- and cold-resistance tests, SPSS 28.0 software was used. A normality test followed by one-way ANOVA and a *post hoc* Tukey test was applied. For the variables not normally distributed, the non-parametric Kruskal-Wallis test was used. Significant differences were considered when $p<0.05$. The results showed that sperm from SR group kept at 5°C for 5 days (81.61±1.08%) or first at 5°C and then at 15°C (81.50±1.58%) have lower viability than those stored at 15°C for 5 days (85.17±1.48%; p=0.024 for 5-group and p=0.020 for 5/15-group) or first at 15°C and later at 5°C (85.22±1.47%; p=0.022 for 5-group and p=0.018 for 5/15-group). Similar pattern was observed for BE group in total motility, meaning there were fewer motile sperm when kept for 5 days at 5°C (85.89±2.52%) than when kept at 15°C (91.83±0.86%; p=0.002) or first at 15°C and then at 5°C (90.67±1.03%; p=0.013). Interestingly, after performing thermal- and cold-resistance test, all the differences between thermal conditions disappeared in both groups (p> 0.05). The findings show that the sperm from both, sperm rich fraction as from the whole ejaculate, can be stored, besides the common temperature at 15°C, using a combination of 15ºC and 5ºC. However, the reverse combination (5/15-group) should be avoided as it results in decreased sperm quality.

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Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY OF REPRODUCTION IN MALE AND SEMEN TECHNOLOGY

Potential sperm freezability biomarkers in young bucks of Cabra Blanca de Rasquera

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Keywords: sperm, freezability, bucks

Potential sperm freezability biomarkers as tools for early breeder selection for the creation and maintenance of sperm cryobanks are not determined in young bucks. Therefore, a retrospective study was performed to relate thawed sperm quality of 6 male donors of the Cabra Blanca de Rasquera breed at the age of 3 years old with melatonin concentration, scrotal circumference (SC) and body weight (BW) at early ages in order to help in the early selection of young males suitable for semen freezing. SC and BW measurements as SC/BW ratio and blood extraction for melatonin determination were performed every month from June to December, when the six males were 9 months old and finished at the age of 15 months. For melatonin determination, a jugular blood sample was collected (before 08.00 h) and immediately centrifuged at 1500× g for 10 min. The harvested serum was then stored at –20oC until hormone analyses. The concentration of melatonin in serum was measured by commercial Goat Melatonin (MT) ELISA Kit. Cat No. MBS267560. The frozen sperm samples (n=6 replicates/male) for this study belong to semen collections taken in autumn by artificial vagina. Then, all individual fresh ejaculates were centrifuged twice and diluted in the extender (15% powdered egg yolk and 5% of glycerol, final concentration), equilibrated for 4h at 5°C and packed into 0.25 mL straws before freezing in liquid nitrogen vapor. After thawing, sperm viability and morphology were assessed using eosin/nigrosin stain evaluating 200 cells/slide and 2 slides/sample. Statistical analyses were performed using SPSS program to compute the significance levels for Spearman correlations. Although significant variation (mean±SD, p<0.05) on melatonin concentration (129.3±33.0, pg/mL) and sperm viability percentage (45.4±11.0, %) between males along the negative photoperiod was observed, no significant correlations were found between thawed sperm quality parameters in adult males and melatonin concentration registered in early ages along the negative photoperiod, only a positive correlation between this hormone concentration and bent tail alive sperm percentage (r=0.38, p=0.021). Also, BW (39.4±3.4 Kg, mean ± SD) in young males showed a positive relation to dead sperm showing bent tail (r=0.39, p=0.028), to alive sperm with bent tail (r=0.48, p=0.003) and to the total percentage of bent tail sperm (r=0.51, p=0.001). Similarly, but negatively, SC/BW ratio was negatively correlated to alive bent tail sperm (r=-0.46, p=0.005), dead sperm with bent tail (r=- 0.37, p=0.027) and total sperm with bent tails (r=-0.53, p><0.001) after sperm thawing. Finally, only one negative correlation was found between SC (23.6±1.6 cm, mean ± SD) in young males and thawed sperm viability (r=-0.37, p=0.027) in adult sperm samples. In conclusion, further studies are needed to find more reliable correlations between young male parameters and thawed sperm quality in this endangered Catalonian local breed. Nevertheless, our results suggest the importance of considering the SC/BW ratio instead to only consider these parameters independently.> <0.05) for melatonin concentration (129.3±33.0, pg/mL) and sperm viability (45.4±11.0, %) was observed between males throughout the photoperiod, no significant correlations were found between thawed sperm quality parameters in adult males and melatonin concentration at an early age. A positive correlation was seen between melatonin concentration and proportion of bent tail live sperm (r=0.38, p=0.021). Also, BW (39.4±3.4 Kg, mean ± SD) in young males showed a positive relation to dead sperm with bent tail (r=0.39, p=0.028), to live sperm with bent tail (r=0.48, p=0.003) and to the total proportion of bent tail sperm (r=0.51, p=0.001). Similarly, SC/BW ratio was negatively correlated to live bent tail sperm (r=-0.46, p=0.005), dead sperm with bent tail (r=- 0.37, p=0.027) and total sperm with bent tails (r=-0.53, p<0.001) after sperm thawing. Finally, only one negative correlation was found between SC (23.6±1.6 cm, mean ± SD) in young males and thawed sperm viability (r=-0.37, p=0.027) in adult sperm samples. In conclusion, further studies are needed to find more reliable correlations between young male parameters and thawed sperm quality in this endangered Catalonian local breed. Nevertheless, our results suggest the importance of considering the SC/BW ratio instead to only consider these parameters independently.> <0.001) after sperm thawing. Finally, a negative correlation was found between SC (23.6±1.6 cm, mean ± SD) in young males and thawed sperm viability (r=-0.37, p=0.027) in adult sperm samples. In conclusion, further studies are needed to find more reliable correlations between young male parameters and thawed sperm quality in this local endangered Catalonian breed. Nevertheless, our results suggest the importance of considering the SC/BW ratio instead of considering these parameters independently.