Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Physiology of reproduction in male and semen technology

Evaluation of Dithiothreitol and Glutathione antioxidants supplemented during cryopreservation of Large White boar semen and subsequent to fertilization of porcine oocytes

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Cryopreservation is the most practical approach for the long-term storage of sperm in boars. However, the freezing and thawing processes result in compromised sperm function and *in vitro* fertilization (IVF) success. The study aimed to evaluate the fertilizing ability of frozen-thawed semen on matured porcine oocytes following IVF. Semen was collected from three Large White boars and then transported to the laboratory for evaluation. Semen was diluted with Beltsville Thawing Solution then equilibrated at 17°C for 120 min, later centrifuged at 800 x g for 10 min at 15°C. Semen pellets were resuspended with egg yolk citrate base extender and placed back for 90 min at 5°C. Semen was supplemented with 4 different fraction B (control, 5 mM DTT, 5 mM GSH and a combination of 2.5 mM DTT + 2.5 mM GSH) extenders and loaded into 0.25 mL freezing straws, then placed in vapour for 20 min, later plunged into nitrogen tank (-196°C). Semen was thawed at 37°C then evaluated for sperm motility. Pig ovaries were collected from the local abattoir and transported to the laboratory within an hour in 0.9% saline water in a thermos flask at 38°C. The slicing method was used to retrieve the oocytes from the ovaries. Oocytes were washed three times in modified Dulbecco phosphate buffered saline and modified Medium 199. Only good quality oocytes were *in vitro* matured for 44 hrs in North California State University-23 medium supplemented with 10 ng/mL of follicle-stimulating hormone, 10 ng/mL of luteinizing hormone and 10% porcine follicular fluid. The oocytes were washed five times in pre-warmed (37°C) 100 µl of IVF medium drops and then distributed into 50 µl of the IVF drops. A drop of 50 µl of capacitated diluted (1 x 10^6) fresh and frozen-thawed sperm was used for IVF. Sperm and oocytes were co-incubated at 38.5°C in a moist atmosphere of 5% CO_2 in the air for 6 hrs. The fertilization rate was evaluated by the presence of a pronucleus with the aid of an inverted microscope using Hoechst 33342 staining. Data were analyzed using the GLM procedure. Treatment means were separated using Fisher's protected t-test least significant difference (LSD) at a 0.05 level of significance. Percentage data are presented as mean ± standard deviation values. The sperm total motility of frozen-thawed semen ranged from 22.4 to 32.0% for all treatments (P>0.05). The percentage of polyspermy differed significantly among the treatments (P<0.05). The total fertilization rate ranged from 31.9 to 48.7%. Raw semen (11.8±9.4) and combination of 2.5 mM DTT + 2.5 mM GSH (14.1±10.4) treatments recorded higher percentage of polyspermy as compared to the 5 mM GSH (0.8±1.9) and 5 mM DTT treatments (2.2±4.9), (P<0.05). The 5 mM DTT treatment (19.7±9.9) non-significantly had a high percentage of oocytes showing normal fertilization (2 PN) as compared to all the treatments. The 5 mM Glutathione treatment (31.9±8.6) non-significantly recorded the least percentage of total fertilization rate as compared to all the treatments (P>0.05). In conclusion, both Glutathione and Dithiothreitol did not have any effect on the fertilization rate by cryopreserved boar semen.

These results are already published https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9104872

Keywords: dithiothreitol, glutathione, semen

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Physiology of reproduction in male and semen technology

The effect of cryoprotectants combination at different concentrations during cryopreservation of semen from windsnyer boars.

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Boar sperm is associated with the post-thawed sperm quality reduction due to the low cholesterol and high polyunsaturated fatty acid contents in their sperm plasma membrane (Li J, 2017, Cryobiology, 80, 119-125). The objective of the study was to investigate the efficacy of the cryoprotectants combination at different concentrations on the Windsnyer boars sperm quality during cryopreservation. A total of 18 ejaculates (6 replications/boar) were collected from three Windsnyer boars of proven fertility with the use of the hand-gloved technique method, twice per week. Boars semen were pooled and was extended with Beltsville Thawing Solution [(BTS) IMV Technologies, France], held at 18°C for 3 hours and centrifuged. The sperm pellet was re-suspended with Fraction A (20% egg yolk + BTS) and cooled at 5°C for 1 hour. Following cooling, semen was divided and diluted into the combination of the cryoprotectants [Ethylene glycol {(EG) Sigma-Aldrich®, Munich, United State of America} + Glycerol {(GLY) Laboratory Consumables & Chemicals Supplies cc, Johannesburg, South Africa} + Propanediol {(PDO) Rochelle Chemicals & Lab Equipment, Johannesburg, South Africa}] at equal contribution to make the total concentrations of 4, 8, 12 and 16% and the 0% (control; without cryoprotectant) 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. However, the conventional boar semen cryopreservation protocol (4% GLY + 20% egg yolk + BTS) was tested, and the results were compared with the current experiment. 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 the Sperm Class Analyser® (Microptin, Spain) system. Eosin-Nigrosin staining was used to evaluate sperm viability and morphology at 100X magnification under a phase-contrast microscope (Olympus, BX 51FT, Tokyo, Japan). A total of 200 sperm per slide/treatment was counted for sperm viability and morphology characteristics. 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 and the significant differences were determined by P-value at a significant level of $P<0.05$. The highest post-thawed sperm total motility (21.4±7.2) percentage was recorded in the treatments supplemented with 16% combination of EG + GLY + PDO, significantly different from 4% GLY (18.0±8.2). The least post-thawed sperm progressive motility percentage was recorded in the treatments supplemented with 0% (1.1±1.3) and 8% (4.7±3.3) combination of EG + GLY + PDO, significantly different from 4% GLY (8.0±6.4). However, semen samples supplemented with a 4% (28.8±4.4) and 16% (30.4±6.2) combination of EG + GLY + PDO recorded the highest post-thawed live normal sperm percentage which was significant to 4% GLY (32.1±6.7). There was no post-thawed sperm abnormality percentage recorded in the semen supplemented with the 0% combination of EG + GLY + PDO ($P<0.05$). The 16% combination of EG + GLY + PDO maintained boars sperm survival during cryopreservation.

Keywords: boars, cryoprotectants, cryopreservation

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Physiology of reproduction in male and semen technology

Impact of diets enriched with omega-3 fatty acids or antioxidants on Belgian blue bull semen

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Improving bull semen quality and quantity is an important issue for artificial insemination (AI) centers. Spermatogenesis is influenced by many factors, including genetics and nutrition. The objective of the study was to evaluate the impact on blood and semen parameters of enriching the diet of Belgian blue bulls with 1. Omega-3 fatty acids: concentrate enriched with extruded linseed (Linamix, Dumoulin) providing an omega-3 concentration of 14g/kg instead of 3.7 and extra vitamin E to prevent lipid peroxidation (160mg/kg instead of 50) = O3 diet; 2. Antioxidants :concentrate supplemented with 47mg/kg beta-carotene, 200mg/kg encapsulated grape extracts rich in polyphenols (Nor-Grape BP-0, Nor-Feed), 600 mg/kg melon extracts rich in superoxide dismutase (Melofeed, Lallemand) and 1mg/kg selenium instead of 0.8 = AX diet; 3. in comparison with the basic concentrate without enrichment = CT diet. After at least one month of receiving the CT diet, 24 Belgian blue bulls (1 to 9 years old) housed in the AI center of Inovéo (Ciney, Belgium) were randomly assigned to 3 groups. Each group received successively the three diets (basic diet + 1.25kg concentrate per 100 kg BW per day) for a period of 4 months in a different order. At the end of each 4-month period, specific analysis were conducted on semen (volume, concentration and computer assisted sperm analysis for motility and morphological parameters; fatty acid profiles analysis using gas chromatography) and blood samples (concentration in selenium, vitamin A and vitamin E). Statistical analysis was performed using a mixed model for repeated measurements with diet, period of the year and their interaction as fixed effects. A majority of the measured parameters was influenced by the period of sampling. As expected, blood concentrations in selenium and vitamin A were on average higher with the AX diet ($p=0.0011$ and 0.034, respectively), while the vitamin E concentrations were higher with the O3 diet by comparison with the two other diets ($p<0.0001$). A higher semen concentration was found with the AX diet by comparison with the CT diet (mean: 1.45 vs 1.28 billion spz/ml; $p=0.037$). The proportion of motile spz also tended to be higher for the AX by comparison to the CT regime (mean: 60.6 vs 56.1%; $p=0.098$). No significant difference was observed for the semen parameters between the AX and the O3 diet or between the O3 and the CT diet. The diet did not significantly influence the other measured sperm characteristics, including the proportion of progressive spermatozoa. The proportion of saturated, monounsaturated or polyunsaturated fatty acids in sperm cells was similar between diets. However, the O3 diet significantly increased the proportion of docosahexaenoic acid (DHA; $p=0.0001$) and its precursor alpha-linolenic acid (ALA; $p=0.0009$) by comparison with the two other diets. A high DHA content in sperm membranes usually correlates with a better sperm quality, which was not demonstrated in the present study. In conclusion, the diet enriched in linseed increased the DHA content of the sperm, while supplementing the diet with a cocktail of natural antioxidants had a positive impact on semen concentration and motility.

Keywords: belgian blue bull, semen, antioxidants

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Physiology of reproduction in male and semen technology

Evaluation of a new sperm purification device for preparing bovine frozen-thawed semen for in vitro fertilization

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Density gradient centrifugation is a common approach for preparing frozen-thawed semen for *in vitro* fertilization. This method is time and labor intensive, requires experience and sperm cell recovery is limited. Therefore, we tested a novel sperm purification device, the VetCount™ Harvester (MotilityCount ApS, Copenhagen, Denmark). The core elements of the VetCount™ Harvester are two chambers which are separated by a microporous membrane with a pore diameter of 10 µM. Spermatozoa are selected when actively swimming out of the semen-filled chamber, i.e., through the membrane, into the other chamber which contains a sperm collection medium. The handling of the VetCount™ Harvester is simple. Semen and collection medium, in this experimental approach a TALP (Tyrode's Albumin Lactate Pyruvate) based medium, are injected into the chambers and, after 30 minutes incubation at 38°C, the medium is aspirated and the spermatozoa are ready for further use. In a preliminary evaluation, we assessed sperm recovery and semen quality of frozen-thawed but otherwise untreated bull semen and frozen-thawed semen treated with the VetCount™ Harvester or BoviPure™ gradient centrifugation (Nidacon International AB, Mölndal, Sweden), a standard technique in our laboratory. Frozen semen samples from six different bulls (n = 6), ten straws of one ejaculate per bull, were analyzed. Sperm concentration was determined using a hemocytometer chamber and the total sperm count was calculated. Motility parameters were assessed using IVOS II, a computer assisted sperm analysis (CASA) system, and flow cytometry was used to simultaneously evaluate viability, acrosome integrity, membrane fluidity and intracellular Ca^{+2} concentration. Results were tested for significant differences using Wilcoxon's signed rank test with Bonferroni correction. A *p*-value of <0.05 was set as significance level. BoviPure™ and VetCount™ Harvester treatment increased the progressive sperm motility compared to frozen-thawed semen samples (82.4±18.3%, 78.8±8.4%, and 41.2±18.4%, respectively; $p<0.05$). The proportion of viable, acrosome intact sperm cells with low intracellular Ca^{+2} concentration and low membrane fluidity was increased after VetCount™ Harvester or BoviPure™ treatment (78.6±6.0%, 76.5±4.4%, and 37.1±13.2%, respectively; $p<0.05$). Following VetCount™ Harvester filtration, viable, acrosome intact sperm cells had a lower normalized intracellular Ca^{2+} concentration (67±10% of the concentration in untreated semen; $p<0.05$) compared to spermatozoa following gradient centrifugation (84±14%; $p<0.05$) or untreated sperm cells (normalized to 100%). There was no significant difference in recovery rate of sperm cells between the VetCount™ Harvester and BoviPure™ treatment (12.4±3.6% and 14.4±5.1%; $p>0.05$). The data demonstrate that the VetCount™ Harvester treatment selects a high-quality fraction of sperm from frozen-thawed bull semen with even lower free intracellular Ca^{2+} concentrations than a BoviPure™ gradient centrifugation. We are currently investigating whether sperm treated with a BoviPure™ gradient or the VetCount™ Harvester differ in cleavage rate, blastocyst rate and quality when they are used in bovine *in vitro* fertilization.

Keywords: cattle, sperm, purification

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Physiology of reproduction in male and semen technology

Characterization of extracellular vesicles in seminal plasma of fertile and subfertile rabbit bucks

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Extracellular vesicles (EVs) 40-120 nm in diameter are secreted particles present in all biological fluids, transporting molecular components that can affect gamete maturation, fertilization, early embryonic development, and embryo-maternal communication. The study of the composition of the EVs present in the seminal plasma (SP) and its correlation with the fertilizing capacity can help us modulate or enhance the productive results of the rabbit bucks. This study aimed to establish a standardized procedure for isolating and characterizing SP-EVs from rabbit bucks of different semen qualities. Fourteen sexual mature rabbit bucks Cal x NZW (10-12 months old) were used. During a month, 2 ejaculates/week were obtained with an artificial vagina to determine their semen quality (CASA, Microptic S.L., Barcelone, Spain), choosing 3 rabbits of high (HSQ) and 3 of low (LSQ) semen quality. A total of 25 rabbit females were artificially inseminated (seminal dose: $5x10^6$ spermatozoa/ml) with the diluted ejaculate of each male, confirming their high or low fertilizing capacity (fertility: 71.9 and 40.9%; prolificacy: 11.6± 0.3 and 8.1±0.4 liveborn, 0.1±0.1 and 0.7±0.1 stillborn in HSQ and LSQ, respectively; $p<0.001$). Then, 6 ejaculates of each animal obtained in 3 weeks (2 ejaculates/week) were centrifugated (800 × g 20 min, 2000 × g 20 min, and 16000 × g 60 min), and the resulting SP was pooled and frozen at −80 °C. The isolation of SP-EVs from ejaculates of each male was based on size exclusion chromatography analysis PURE-EV® (Hansa BioMed Life Sciences). SP-EVs were quantitative and qualitatively characterized by transmission electron microscopy (Jeol, Ltd Tokyo, Japan), nanoparticle tracking analysis (Nanosight NS500: Marven, INC) using software NTA 3.1, and western blot to confirm the expression of the classical exosome markers (HSP70, CD9 y ALIX). A correlation analysis between seminal parameters and the concentration and size of SP-EVs from the two groups of males was made (SAS, 2001). Different SP-EVs concentrations ($8.53x10^{11}$±$1.0x10^{11}$ and $1.84x10^{12}$±$1.75x10^{11}$ particles/ml of SP; p=0,008) with a similar average size (143.9 ± 11.9 and 115.5 ± 2.4 nm; p=0.7422) in HSQ and LSQ males, respectively were observed. The concentration of SP-EVs was positively correlated with the percentage of abnormal forms ($r=0.94$; $p<0.05$) and with the percentage of immotile spermatozoa ($r=0.88$; $p<0.05$). Particle size was not correlated with any kinetic parameter. We can conclude that the methodology used for the extraction and characterization of the SP-EVs is valid by confirming their existence in the SP of rabbits and the SP-EVs concentration depends on semen quality and its fertilizing ability. *Supported by projects RTI 2018-094404-B-C-21 and PID2019-111641RB-I00.*

Keywords: extracellular vesicles, semen quality

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Physiology of reproduction in male and semen technology

Bovine oviductal fluid, the physiological additive for bovine sperm selection.

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Today, during a cycle for IVP of bovine embryos, the current blastocyst yield per oocyte is similar to the figures reported a decade ago, with only 30-40% of the collected oocytes reaching the blastocyst stage (Lonergan et al., Theriogenology, 81(1), 49-55, 2014). Since bovine oviductal fluid (BOF) is the physiological environment in which the last stages of capacitation and fertilization take place, we hypothesized that the inclusion of BOF into the composition of the sperm preparation medium might select a sperm population of better quality in terms of motility, viability and capacitation status, which will contribute to increasing the efficiency of the IVP process. Sperm quality was evaluated in terms of motility, viability, apoptosis, plasma membrane fluidity, and state of the acrosome. Asturian Valley bull sperm straws were thawed at 38 °C for 30 s. Each replicate (N=5) consisted of 4 straws from the same bull and a different bull was used in each replicate. BOF from the late follicular phase was acquired from EmbryoCloud (Murcia, Spain). Spermatozoa were selected by the swim-up method (Ruiz et al., Reproduction in Domestic Animals, 48(6) e81-e84, 2013). Two different media were used: Swim-up BSA, containing 6 mg/ml BSA (Parrish et al., Biology of Reproduction, 40(5), 1020-1025, 1988); and Swim-up BOF, where BSA was replaced by 1% BOF (v/v). To analyse sperm motility, sperm samples were evaluated at 38 ºC and 200X magnifications under a negative phase-contrast microscope coupled to a CASA system (ISASv1, ProiserR+D, Valencia, Spain). To evaluate viability, acrosomal integrity, and membrane fluidity of the samples by flow cytometry, the sperm concentration was set at $2x10^5$ spermatozoa per mL and the sperm suspension was incubated for 15 min at 37 °C with the following combinations of fluorochromes: i) 2.5 µg/mL propidium iodide and 1 µg/mL *Pisum sativum* lectin I conjugated with fluorescein isothiocyanate to assess sperm viability and acrosome integrity; and ii) 2.7 mM merocyanine-540 and 25 nM Yo-Pro1 to assess sperm viability and plasma membrane fluidity. Subsequently, samples were subjected to analysis by a Guava Easycyte 6-2L flow cytometer (Merck Millipore, Hayward, USA). Data, presented as mean ± SEM, were analyzed by the Student's t-test using the IBM SPSS Statistics package (IBM, Armonk, USA). Differences were considered significant when $P < 0.05$. BOF enabled a higher ($P < 0.01$) total and progressive motility, and higher kinematic parameters after swim-up, except for ALH and BCF. The percentages of spermatozoa viable, non-apoptotic and with non-reacted acrosome were similar in both groups. The proportion of spermatozoa with high membrane fluidity was higher after swim-up in BOF (37.5 ± 4.2%) than in BSA (21.9 ± 1.6%; $P < 0.05$). To sum up, the inclusion of BOF into the composition of the sperm preparation medium selects a population of functional spermatozoa with improved motility, which might contribute to increasing the fertilizing ability of impaired semen samples and so, enhance the efficiency of the IVP of bovine embryos. Supported by Fundación Séneca, Región de Murcia, Spain (21651/PDC/21).

Keywords: Oviductal fluid, bovine, spermatozoa

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Physiology of reproduction in male and semen technology

The fate of porcine sperm CRISP2 from the perinuclear theca before and after in vitro fertilization.

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In a previous study (Zhang et al., Biol Reprod 105:1160-1170; 2021) we reported that porcine sperm cysteine rich secretory protein 2 (CRISP2) is localized in the post-acrosomal sheath (PAS)-perinuclear theca (PT) as reduction-sensitive oligomers. In the current study, the decondensation and removal of CRISP2 was investigated during *in vitro* sperm capacitation, both after induction of the acrosome reaction and after *in vitro* fertilization. Confocal immunofluorescent imaging revealed that additional CRISP2 fluorescence appeared on the apical ridge and on the equatorial segment (EqS) of the sperm head following capacitation, likely a result of the local de-oligomerization of CRISP2. After an ionophore A23187 induced acrosome reaction, CRISP2 immunofluorescence disappeared from the apical ridge and the EqS area partly due to the removal of the acrosomal shroud vesicles but also partly due to its presence in a subdomain of EqS (EqSS). The fate of sperm head CRISP2 was further examined post-fertilization. *In vitro* matured porcine oocytes were co-incubated with boar sperm cells for 6-8 h and the zygotes were processed for CRISP2 immunofluorescent staining. Notably, decondensation of CRISP2, and thus of the sperm PT, occurred while the sperm nucleus was still fully condensed. CRISP2 was no longer detectable in fertilized oocytes in which sperm nuclear decondensation and paternal pronucleus formation was apparent. These data indicate that PT decondensation and degradation may be executed in advance of sperm DNA decondensation post-fertilization. This rapid dispersal of CRISP2 in the PT is likely regulated by redox reactions for which its cysteine rich domain is sensitive. Reduction of disulfide bridges within CRISP2 oligomers may be instrumental for PT dispersal and PT elimination. These results raise important questions such as whether the dispersed PT proteins in the oocyte cytoplasm may be involved in oocyte activation, as well as in male nuclear chromatin decondensation in order to form the male pronucleus.

Keywords: CRISP2; porcine; sperm; perinuclear theca; decondensation; fertilization; oocyte.

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Physiology of reproduction in male and semen technology

Genomic analysis of bovine beta-defensin genes implicated in sperm function fertility

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Beta-defensins are short secreted peptides that have traditionally been characterised as antimicrobial. However, it is known that some beta-defensins have important functions in fertility. For example, beta-defensin 126 is an important component of the sperm glycocalyx and is involved in capacitation of sperm prior to fertilisation in macaques, and promotes sperm motility in cattle. Genes encoding beta-defensins are known to show extensive deletion and duplication (known as copy number variation (CNV)) in cattle and other mammals, including humans. However this has not been characterised in detail in cattle. In this study we mapped publicly available sequencing data (1000 Bulls Consortium) from 100 bulls of different breeds to the bovine genome (ARS-UCD1.2-bosTau9) assembly based on long read PacBio sequencing, which is likely to have a more accurate assembly of complex repeated regions of the genome, such as the beta-defensin regions. We show that the chromosome 13 beta-defensins, including beta-defensin 126, are commonly duplicated in Holstein bulls. Other beta-defensins on chromosome 27, in particular DEFB103, show extensive copy number variation across breeds, including complete loss of the gene. In addition we selected positive control bulls target that high and low copy numbers associated with beta-defensin by the mapping. We are currently testing an extended fertility panel of Holstein bulls and the positive control bulls for beta-defensin genes showing CNV using digital droplet PCR, with the aim of investigating the relationship between CNV at bovine beta-defensin genes and fertility.

Our results may improve our understanding of CNV, which is known as an important genomic structural variation in cattle. We also believe that beta-defensin will provide important information about potential CNV effects on reproductive performance, which forms the basis for its inclusion in a future dairy cattle breeding programme.

Keywords: CNV, Beta-defensin, Fertility, Cattle