A173E Physiology of reproduction in male and semen technology

ADAM protein expression in avian sperm and female genital epithelial cells: relation to sperm storage

L. Cordeiro[1], C. Riou[1,2], N.Gérard[1]

[1]PRC, INRA, UMR INRA85, UMR CNRS 7247, Université de Tours, IFCE, Nouzilly, France; [2]ALLICE, Station de Phénotypage, Nouzilly, France.

Keywords: ADAM protein, sperm storage tubes, chicken.

The cell-cell interaction can be mediated by the interface of a protein-protein. "ADAM is a transmembrane protein that contains a disintegrin and metalloprotease domain and, therefore, potentially has cell adhesion and protease activities" (Primakoff & Myles, Trends Genet., 2000, 16 (2): 83 -7). Avian sperm storage tubes (SST) are uterine-vaginal junction (UVJ) epithelial structures that can store spermatozoa (spz) through an interaction mechanism that is not yet clearly understood. This study was devoted to investigating the presence or absence of ADAM proteins in chicken SST as a model of sperm-epithelial cell adhesion and interaction. For *in vitro* tests, 3 hens per experiment (2 triplicate experiments, n = 18 hens) were euthanized and the internal epithelium UVJ was collected, digested (1 µg / ml collagenase) and the SST fragments were isolated by Percoll gradient column. SST (100/well), sperm (25.10^4 sperm/well) cultures and co-culture SST + spz (25.10^4 sperm / 100 SST) were grown in DMEM 199 medium supplemented with 10% BFS and gentamycin at 37°C, 5% CO2 atmosphere at 3 times: 0, 2 and 24h. For *in vivo* tests, 3 other hens were inseminated with 200 x 10^6 spz (pool of 6 roosters) and euthanized 24 hours later, while 3 other non-inseminated were used as control. In all experiments, antibody anti-ADAM-1-CT rabbit peptide sequence was used: CSSPGSGGSVDSGP (C-terminal part, close to the transmembrane metalloprotease domain), as described by Fàbrega et al. (Reproductive Biology and Endocrinology, 2011, 9:96). UVJ were isolated and prepared for immunohistochemistry (IHC). The slides were incubated overnight at 4°C with the primary antibody and incubated for 30 min with the secondary antibody ImmPRESS^{TM} HRP Anti-Rabbit/Mouse IgG (Vector) and revealed with peroxidase. Protein concentration was determined individually in the samples, and the pools were prepared with equal protein concentration of each sample. Ten µg of protein in Laemmli buffer (5v:1v) were loaded on SDS-10% PAGE, and then transferred to nitrocellulose filters. The membranes were incubated with anti-ADAM-1 antibody overnight in blocking solution (5% non-fat dry milk in TBST [10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4]). The ADAM peptide exhibited sequence homology with *Gallus gallus* ADAM-12 and ADAM-33 (www.clustal.org/ multiple alignment program). ADAM was detected as a single 90 kDa band in SST samples and 3 bands (90, 45 and 40 KDa) in spz samples. Signals decreased in SST + spz co-culture in the cells and the respective supernatant, but a 90 kDa band was observed in the supernatant after 24h co-culture. Anti-ADAM IHC UVJ slides showed positive staining for spermatozoa located in the SST lumen and in small vesicles on apical SST cells. We can conclude that ADAM proteins are present in SST epithelial cells and can be released in the presence of spermatozoa, probably modulated by cell-cell interaction.

Supported by Université François Rabelais de Tours and Région Centre, France.

A174E Physiology of reproduction in male and semen technology

Analysis of zona pellucida binding properties of boar sperm subpopulations separated by carbohydrate affinity

L. González-Brusi, R. López-Úbeda, J.G. Hamzé, P. Cots, J. Ballesta, M. Jiménez-Movilla, M.J. Izquierdo-Rico, M. Avilés

Department of Cell Biology and Histology, Faculty of Medicine, University of Murcia, IMIB-Arrixaca, Campus Mare Nostrum, Murcia, Spain.

Keywords: carbohydrate affinity, flow cytometry sorting, ZP binding assay.

In mammals, sperm-sugar interaction is involved in relevant biological processes such as the sperm binding to the zona pellucida (ZP) and the formation of the sperm oviductal reservoir. The aims of this study are the identification of sperm subpopulations based on their affinity for carbohydrates, the isolation of these sperm subpopulations and the characterization of their ZP binding properties. Ovalbumin (OVA) which has high-mannose type glycans and the Lewis^a epitope (Le^a), consisting of Galβ1-3[Fucα1-4]GlcNAcβ, were tested. Heterospermic sperm samples from boars of proven fertility were coincubated with OVA-Alexa594 or Le^a-FITC for 30 minutes in PBS with 1mM sodium pyruvate. Subpopulations separation was done in a Sony SH800Z flow cytometer with excitation lasers at 488 nm and 561 nm, sorting 3500-4000 cells/second. Sperm were collected in tubes containing 100µl of TEST- 2% egg yolk buffer to minimize oxidative damage. Sperm resuspended in capacitating TALP medium at a concentration of 10^5 sperm/ml were incubated with 50 intact isolated ZP (n = 3). Isolated ZP were obtained form porcine ovaries collected at a local slaughterhouse. Cumulus-oocyte complexes were taken from antral follicles and treated with hyaluronidase. ZP were isolated with very thin pipettes in PBS containing protease inhibitors. After 2 h of incubation at 37ºC and 5% CO_2, samples were washed twice to detach weakly bound spermatozoa, fixed in PBS with glutaraldehyde 0.5% for 30 min and stained with Hoechst 33342. Spermatozoa bound to each ZP were evaluated with an Olympus IX70 fluorescence microscope. A Student's test was used to compare between each pair of samples and differences were considered significant when $P < 0.01$. Percentages of spermatozoa from each subpopulation; Le^a-, Le^a+, OVA- and OVA+ were 77%, 18%, 82% and 14% respectively. A 4-5% of spermatozoa between each positive and negative subpopulation were discarded. The number of sperm bound to the ZP of the Le^a-, Le^a+, OVA- and OVA+ sperm subpopulations were 51.8 ± 22.5, 18.9 ± 8.8, 58.1 ± 32.3, 17.5 ± 6.8. Two different controls were perfomed. A total of 113.1 ± 32.4 spz/ZP and 38.2 ± 14.1 spz/ were observed for untreated ejaculated spermatozoa and sorted sperm not labelled. In both Le^a and OVA, ZP binding was significantly higher in the negative sperm subpopulations compared to the positive ones. Le^a+ subpopulation showed a high percentage of non viable spermatozoa (40 ± 10.6%, n = 4), as assessed with propidium iodide, which correlates to less sperm bound to the ZP, while Le^a- viability was over 90%. Our results showed the existence of different sperm subpopulations with a different affinity for carbohydrates. These sperm subpopulations had dissimilar affinity for the ZP and could have a different fertilization ability. The biological significance of these sperm subpopulations will be analyzed in *in vitro* fertilization assays and binding to the oviductal epithelial cells.

Supported by MINECO-FEDER (AGL2015-70159-P).

A175E Physiology of reproduction in male and semen technology

Sperm motility in thawed bull semen is increased by a short incubation before analysis

I.B. Lima-Verde, T. Ntallaris, J.M. Morrell

Swedish University of Agricultural Sciences, Uppsala, Sweden.

Keywords: incubation time, cryopreservation of semen, sperm motility.

Cryopreservation of semen is a widely used technique, mainly to provide samples with certified origin and quality for use in artificial insemination (AI) in dairy cattle. After thawing, spermatozoa may require a period to recover their proper motility, which is a factor directly related with the semen quality. Our aim was to determine the effects of a short incubation on total and progressive motility after thawing bull semen. The samples were collected from 12 bulls at a commercial bull station (Viking Genetics, Skara, Sweden) and were extended in OptiXcell® (IMV Technologies, L´Aigle, France) to provide a sperm concentration of 69×10^6 spermatozoa/mL. All samples were frozen in 0.25 mL plastic straws and stored in liquid nitrogen. The straws were thawed at 37°C for 12 s and the semen content was split into four tubes and incubated at 38°C for 0, 5, 10 or 15 min. Sperm motility assessment was performed by computer assisted sperm analysis (CASA – SpermVision, Minitub GmbH, Tiefenbach, Germany), connected to a microscope (Olympus, Tokyo, Japan) with a heated stage (38°C). Aliquots of 5µL from the thawed sperm samples were placed on a warm glass slide with a coverslip. At least 1000 spermatozoa were analyzed in a total of eight fields using the software program (SpermVision) with settings adjusted for bull spermatozoa. Statistical analysis was performed with SAS (version 9.3), using the proc mixed procedure for linear mixed models. Scheffe's adjustment was used for multiple-post ANOVA comparisons. Results are presented as LSMeans ± standard error of means (SEM). An increased incubation time was associated with increased total motility (0 min 44.7 ± 4.45%; 5 min 47.0 ± 4.45%; 10 min 49.0 ± 4.45; 15 min 56.9 ± 4.45) and progressive motility (0 min 41.5 ± 4.34%; 5 min 43.8 ± 4.34%; 10 min 45.9 ± 4.34; 15 min 53.5 ± 4.34). Motilities were significantly greater after 15 min incubation in OptiXcell® than after 0 min ($P < 0.05$). In conclusion, total and progressive motilities may be increased by incubation before analysis, depending on the extender used. This effect should be remembered when comparing sperm handling treatments, or to avoid introducing bias when multiple samples are to be analysed.

I Lima-Verde received a fellowship from Brazilian Council of Research (CNPq-Brazil, 204411/2014-9). The project was funded by a project grant from the Swedish Farmers´ Foundation (SLF; H13300339) awarded to JM Morrell.

A176E Physiology of reproduction in male and semen technology

Effect of cholescytokinin (CCK) protein on the motility of porcine sperm

R. López-Úbeda[1], FA. García-Vázquez[2], M. Balastegui-Alarcón[1], P. Cots[1], M. Avilés[1], MJ. Izquierdo-Rico[1]

[1]Department of Cell Biology and Histology, Faculty of Medicine, University of Murcia and IMIB-Arrixaca, Murcia, Spain; [2]Department of Physiology, Veterinary School, Campus Mare Nostrum, University of Murcia, and IMIB-Arrixaca, Murcia, Spain.

Keywords: spermatozoa, cholecystokinin, motion parameters.

The expression of cholecystokinin (CCK) gene has been demonstrated in the testis of several different species and CCK-like peptides have been found in spermatocytes and spermatids of the mouse, rat, and monkey (Persson, PNAS; 86: 6166, 1989). In a recent study, Zhou et al suggested its possible involvement in the regulation of protein tyrosine phosphorylation by modulation the uptake of HCO3- and demonstrated the presence of CCK receptors on sperm surface (Zhou, Reproduction; 150:257, 2015). In addition, our group has detected CCK in the porcine oviduct using microarray technology, real-time RT-PCR and immunohistochemical analyses (Acuña, Reprod Fertil Dev; 29: 2387, 2017). Therefore, the aim of this work was to analyse the effect of CCK on sperm motility. Spermatozoa from fertile boars (15x106/ml) were exposed to different CCK (Bachem, Bubendorf, Switzerland) concentrations according to the bibliography (25 μM and 50 μM) in a capacitating medium (TALP) for 1h at 38.5ºC. TALP medium had a bicarbonate concentration of 5 mM and the incubator was adjusted to 1% CO2 according to the Henderson-Hasselbalch equation. Moreover, a control group was incubated under the same conditions without CCK protein. Motion parameters (total motility (%) and progressive motility (%)) were determined using a CASA system (ISAS®, Proiser, Valencia, Spain) after of incubation period. For statistical analysis the mean measurements of CASA for each male (n = 5) were entered into the ANOVA model and compared by post hoc test (Tukey). Differences were considered statistically significant at $P < 0.05$. The results showed that incubation with CCK increased the percentage of total sperm motility (Control: 53.8 ± 2.8%, 25 μM: 59.4 ± 2.1% and 50 μM: 65.4 ± 4.5%) with statistically significant differences between the three experimental groups. Moreover, an increase in the percentage of progressive sperm motility was observed (Control: 34.4 ± 3.9%, 25μM: 38.4 ± 4.8% and 50 μM: 43.2 ± 5.0%) showing significant differences between the control and 50 μM CCK. In both cases the effect of the protein is dose-dependent. No statistical differences were found between the study groups for the rest of motion parameters. In conclusion, these data could suggest a possible implication of CCK protein in the improvement of the sperm motility in the oviduct before fertilization. More analyses are necessary for clarify the molecular mechanism of this protein.

Supported by MINECO-FEDER (AGL2015-70159-P) and Fundación Séneca (19357/PI/14).

A177E Physiology of reproduction in male and semen technology

Porcine periovulatory oviductal fluid decreases the occurrence of Protein Kinase A (PKA) substrates trough sAC/cAMP/PKA during mouse sperm capacitation

C. Matás[1,2], M.G. Gervasi[3], C. Soriano-Úbeda[1], E. París-Oller[1], P.E. Visconti[3]

[1]Department of Physiology, Veterinary Faculty, University of Murcia, Murcia, Spain; [2]Institute for Biomedical Research of Murcia (IMIB), Murcia, Spain; [3]Department of Veterinary and Animals Sciences, University of Massachusetts, Amherts, Massachusetts, USA.

Keywords: oviductal fluid, spermatozoa, phosphorylation.

Several studies have identified important factors involved in the regulation of sperm capacitation, a physiological process necessary to achieve fertilization competence. These events are regulated through activation of the cyclic AMP (cAMP)/protein kinase A (PKA) pathway. On the other hand, porcine oviductal fluids (POF) decreases PKA activity of porcine sperm during capacitation; however, the molecular mechanism of this regulation remains unknown. The aim of this study was to evaluate if porcine periovulatory oviductal fluid (POF) regulates PKA activity during *in vitro* sperm capacitation in mice. POF was obtained from porcine oviducts (n = 40) close to ovulation and it was frozen at -80ºC before use. Sperm samples were obtained from mice cauda epididymides and incubated for 1 hour in: i) non-capacitation media, ii) capacitation media, iii) capacitation media supplemented with 1% POF, iv) capacitation media supplemented with 1% POF and 100 µM of 3-isobutyl-1-methylxanthine (IBMX), which inhibit adenosine 3',5'-cyclic monophosphate phosphodiesterase (cAMP PDE), v) capacitation media supplemented with 1% POF, and 1 mM dibutyryl-cAMP (db-cAMP), that mimics the action of endogenous c-AMP and vi) capacitation media supplemented with 1% POF, 100 µM of IBMX and 1 mM db-cAMP. The protein phosphorylation pattern on PKA substrates was evaluated by Western blotting (WB). Proteins were separated by electrophoresis on 8% SDS-polyacrylamide gels and electrotransferred to PVDF membranes. The latter were treated with the following antibodies: rabbit monoclonal antibody anti-phosphorylated protein kinase A substrates (1:10000) followed by goat anti-rabbit IgG-HRP (1:10000). After developing, the relative amount of signal was quantified by optical densitometry using ImageJ software. Data were analyzed by two-way ANOVA and Tukey post-hoc test ($P < 0.05$). Our results indicate that in the presence of POF, spermatozoa showed a lower PKA substrates phosphorylation pattern than those incubated in capacitation media alone ($P < 0.05$). The effect of the POF was reversed by the presence of db-cAMP and/or IBMX in the media. Besides, our results indicate that POF decreases the sAC/cAMP/PKA pathway during sperm capacitation and that this is not a specie-specific phenomenon. These effects were not observed under non-capacitating conditions. We identified one of the mechanisms by which POF regulates sperm capacitation. This finding would help to understand the oviductal physiology during gamete interaction and could explain some cases of idiopathic infertility. Further studies are required to evaluate whether FOP regulates PKA-related molecular pathways during sperm capacitation in other species such humans.

MINECO-FEDER (AGL 2015-66341-R) and Programa “Salvador de Madariaga” (Ministerio de Educación Cultura y Deporte).

A178E Physiology of reproduction in male and semen technology

Effect of swim-up on the activation of apoptosis in frozen bovine semen: A flow cytometric study

M.M. Silva, L. Soares, F.M. Silva

University of the Azores, Faculty of Agrarian Sciences and environment, Angra do Heroismo, Portugal.

Keywords: bovine sperm, swim-up, flow cytometry.

Techniques universally used to prepare bovine spermatozoa for *in vitro* fertilization (IVF) include the swim-up (SU) method and differential density gradient centrifugation, among others. Although it is clear that not all of them have comparable efficiency in selecting high quality sperm, the use of these techniques can be harmful for sperm quality. The aim of the present study was to detect the sub lethal changes in bull spermatozoa immediately post-thawing (PT) and after SU, namely the levels of apoptotic sperm cells, to assess if the sperm treatment before IVF induces apoptosis in bull spermatozoa. For such purpose, three semen straws per bull from six bulls were thawed by placing them in a water bath (37°C) for 30 sec. Then, sperm apoptosis level was analyzed immediately PT and after the SU procedure using a flow cytometric method by detecting the phosphatidylserine translocation across the plasma membrane using a fluorescein-labeled Annexin-V and propidium iodide (PI). By using these two dyes, four different subpopulations of sperm were observed: a population of apoptotic sperm, a population of early necrotic sperma, a population of necrotic sperm and a population of fully viable sperm cells. The most consistent effect observed was a significant increase ($P < 0.05$) in the fraction of apoptotic sperm (Annexin-V $^{+}$, PI^{-}) after the SU treatment among the six bulls tested. The highest increase of apoptotic spermatozoa after SU was observed on bull four with an increase from 7.0% ± 0.9 to 59.2% ± 0.2, respectively after thawing and after SU. On average, including all bulls, apoptotic values raised from 11.8% ± 3.3 to 16.9% ± 6.5, respectively before and after SU, proving that the bulls presented different values in the activation of apoptosis during the SU process. In addition, the proportion of necrotic sperm (Annexin V^{-}, PI^{+}) was also significantly different among bulls. In particular, bull four, which had the lowest proportion of necrotic sperm, approximately 2% and 4% for PT and SU, respectively. The percentage of viable sperm (Annexin V^{-}, PI^{-}) was significantly different ($P < 0.05$) among bulls. Bull 5 had the highest ($P < 0.05$) proportion of viable sperm in the sperm samples immediately PT. Overall, PT treatment had no significant effect in the number of viable spermatozoa. The present study indicates that the SU technique can have an adverse effect on the spermatozoa membrane stability leading to different degrees of apoptosis in sperm during SU. Apoptotic markers found in ejaculated spermatozoa may represent an important tool for the study of male infertility, and combine with IVF, may be a valuable laboratory routine technique.
Authors are partially supported by the project BEMP-ET, financed by the European Union and the Government of the Azores – Grant "ACORES-01-0145-FEDER-000026.

A179E Physiology of reproduction in male and semen technology

Progesterone induces sperm release from bovine oviductal epithelial cells by modification of the sperm protein and lipid compositions

M. Ramal Sanchez[1,2], G. Tsikis[1], M.C. Blache[1], V. Labas[1,3], X. Druart[1], N. Bernabò[2], P. Mermillod[1], M. Saint-Dizier[1]

[1]INRA-UMR PRC, INRA 85, CNRS 7247, Université de Tours, IFCE, Nouzilly, France; [2]Università degli Studi di Teramo, Teramo, Abruzzo, Italy; [3]Plate-forme de Chirurgie et d'Imagerie pour la Recherche et l'Enseignement (CIRE), Pôle d'Analyse et d'Imagerie des Biomolécules (PAIB), INRA, CHRU de Tours, Université de Tours, Nouzilly, France.

Keywords: progesterone, BOEC, sperm.

After mating or insemination, spermatozoa reach the oviduct where they bind to oviductal epithelial cells (OEC) for hours to days in the so called "functional sperm reservoir" before moving towards the fertilization site. During this storage, the interactions between spermatozoa and OEC are believed to play an important role in sperm selection and capacitation. Recently, after measuring progesterone (P4) concentrations in the post-ovulatory bovine tubal fluid (Lamy et al. Theriogenology 86:1409-1420, 2016) our group evidenced that P4(100 ng/mL) was able to trigger sperm release from bovine OEC (BOEC) *in vitro*, similar to what occurs *in vivo*, selecting a population of spermatozoa with a higher fertilizing competence (Lamy et al. Reproduction 154:497-508,2017). The aim of this study was to elucidate the underlying mechanisms of action. Frozen-thawed bovine spermatozoa ($4x10^6$/mL), after Percoll density gradient (45/90%), were incubated *in vitro* with confluent BOEC for 30 min (humidified atmosphere, 5% CO_2, 38.8°C). After collecting unbound spermatozoa (UnS), P4 was added to the sperm-BOEC culture for 1h. Then, released spermatozoa (P4-ReS) were collected. A group of spermatozoa was similarly manipulated without BOEC nor P4 (CTRL) and another was treated with P4 without BOEC (P4-CTRL). Proteomic and lipidomic profiles were assessed on Intact Cells by Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (ICM-MS) for each group. The presence of Binder of Sperm Proteins (BSP)-1, -3 and -5, the most important proteins involved in sperm-BOEC binding, was studied by Western-Blotting. Fluorescence Recovery After Photobleaching (FRAP) analysis coupled with confocal microscopy were performed on P4-ReS and CTRL groups to evaluate the changes in membrane fluidity, event directly related to capacitation ($n \geq 3$). In total, 139 m/z (mass/charge) peaks were found as differential m/z on spermatozoa proteomic profiles by ICM-MS. The number of differential peaks was highest between P4-ReS and CTRL (97), followed by P4-CTRL vs. CTRL (61), and UnS vs. CTRL (33). By contrast, only 37 peaks were found as differential m/z on lipidomic profiles, all of them from P4-ReS vs. CTRL (33) and UnS vs. CTRL (32) comparisons. A 3 to 4-fold decrease of BSP-1, -3 and -5 was seen on P4-ReS compared to CTRL ($P < 0.05$), but not in UnS and P4-CTRL groups. Lastly, FRAP analysis showed a higher membrane fluidity on P4-ReS compared to CTRL (KW, $P = 0.014$). In conclusion, these results show that binding to BOEC and then P4-induced release from BOEC triggered major changes in sperm protein and lipid composition whereas P4 by itself had a moderate effect. The UnS displayed an intermediate level of changes in proteins and lipids, suggesting a "BOEC effect" that may be due to a short time binding-release process. A loss of BSPs at the surface and an increase in membrane fluidity were evidenced on P4-ReS, suggesting a membrane destabilization probably involved in the increase of fertilizing competence of this sub-population.
MRS research was supported by MSC ITN REPBIOTECH675526.

A180E Physiology of reproduction in male and semen technology

Motility and atomic force microscopy observation of avian spermatozoa incubated in uterine fluid

C. Riou[1,2], L. Cordeiro[1], A. Chaiyasitdhi[3,4], O. Srikimkaew[5], S. Suebka[5], L. Nadal-Desbarats[6], W. Kundhikanjana[5], N. Gérard[1]

[1]PRC, INRA CNRS IFCE Université de Tours, F-37380 Nouzilly; [2]ALLICE, Station de Phénotypage, F-37380 Nouzilly; [3]Biological Engineering Program, KMUT, Bangkok, Thailand; [4]present address: Institut Curie, UMR168, F-75005 Paris; [5]School of Physics, Suranaree University of Technology, Nakhon Ratchasima, Thailand; [6]U930, INSERM & PPF ASB, Université de Tours, F-37044 Tours.

Keywords: spermatozoa, atomic force microscopy, avian species.

In hens, spermatozoa are stored in the genital tract for long periods, and fertilized eggs are produced for up to 3 weeks after insemination. Storage mechanisms and oviducal environment impact on sperm are largely unknown. The aim of this study was to evaluate the effect of uterine fluid (UF) on semen using two different lines of hens that display a long (21 days, F+ line) or a short (10 days, F- line) period of sperm storage. UF from 3 hens of each line was collected 10h after oviposition and pooled. Fresh ejaculates (pool from 6 roosters) were used in both experiments. For the first one, spermatozoa were incubated *in vitro* in either PBS alone, PBS containing 25% or 50% UF, or in pure UF from both lines for 5 min, 1, 2.5, 5 and 24h at 4°C (3 replicates). For the second experiment, UF from both lines were depleted from proteins >3kDa (d-UF) using Vivaspin 500. Spermatozoa were incubated *in vitro* in PBS, d-UF, and pure UF for 5 min, 1 and 2.5h at 4°C (3 replicates). Sperm motility was assessed using computerized method at 41°C (HTM-IVOS II). Metabolites contained in d-UF from both lines were quantified by NMR and sperm morphology was analyzed using Atomic Force Microscopy (AFM). Wilcoxon test was used to compare sperm motility at the different time point. ANOVA analysis was performed to compare sperm motility between the two chicken lines. Welch t-test was used to compare differential metabolites between the two chicken lines. We observed that pure UF and d-UF improve sperm motility compared to PBS ($P < 0.05$). Nevertheless the effect of d-UF was lower than pure UF. In both experiments, sperm motility was higher after incubation in UF or d-UF from F-line than F+ line ($P < 0.05$). NMR analysis reveals that the concentrations of 5 metabolites were higher in d-UF from F+ than F-line ($P < 0.05$), including alanine, succinate, dimethylamide and N-acetyl groups. Moreover, AFM analysis clearly showed an alteration of head morphology of spermatozoa incubated with d-UF from the F- line. This study clearly demonstrates the major role of UF proteins >3kDa on the sperm motility. Nevertheless, the UF fraction (<3kDa) which mainly contains peptides and metabolites improves sperm motility and leads to ultrastructural modification of spermatozoa. Our findings demonstrate that the microenvironment and complexity is a key element during sperm storage.
Supported by Université François Rabelais de Tours and Région Centre, France.

A181E Physiology of reproduction in male and semen technology

Sperm selection by density-gradient centrifugation of Merino ram semen cold-stored up to 48 h improves viability and membrane integrity

D.A. Galarza[1,2], M. Ladrón de Guevara[1], P. Beltrán-Breña[1], D. Rizos[1], A. López-Sebastián[1], J. Santiago-Moreno[1]

[1]Department of Animal Reproduction, INIA, Madrid, Spain; [2] Faculty of Agriculture Sciences, University of Cuenca, Cuenca, Ecuador.

Keywords: sperm, cold-storage, selection.

Liquid ram semen stored at 5°C would be more competent than frozen/thawed for sheep crossbreeding programs. The aim was to evaluate the kinetics and membrane integrity of Merino ram semen cold-stored up to 48h at 5ºC before and after density-gradient centrifugation (DGC) selection. Pools of 3 normospermic Merino ram (2-7 years) ejaculates were collected by artificial vagina in fifteen sessions (45 ejaculates), diluted to $200x10^6$ spermatozoa/ml with skim milk-based extender contained 6% egg yolk and cold-stored up to 48h at 5ºC. Motile spermatozoa were separated by BoviPure® DGC (Galarza et al., 2018, Anim Reprod Sci 192: 261-270) using 250µl of fresh (n = 30) and cold-stored semen (24h: n = 10 and 48h: n = 10). The final pellet of 300µl was used to assess semen quality. The kinetic parameters were evaluated by computer-assisted sperm analysis (CASA) while plasma, acrosomal and mitochondrial membrane status was analyzed by PI/FITC PNA/Mitotracker fluorescence. The effects of storage time (fresh, 24 & 48h) and sperm selection process were analysed by univariant ANOVA and Bonferroni´s test ($P < 0.05$). In terms of sperm storage time, CASA analysis of non-selected semen samples showed a significant decrease after storage for 24 and 48h compared to fresh samples with regard to progressive motility [SPM (%): 52.30 ± 4.1 and 36.9 ± 5.5 vs 71.3 ± 1.6], straight line velocity [VSL (µm/sec): VSL 132.2 ± 6.1 and 109.7 ± 6.3 vs 176.7 ± 4.3], linearity [LIN (%): 69.2 ± 3.5 and 59.0 ± 5.0 vs 82.0 ± 1.2], and straightness [STR (%): 75.7 ± 3.3 and 66.0 ± 4.3 vs 86.9 ± 0.9], respectively. However, analysis of DGC-selected semen showed a decrease only at storage for 48h when compared to 24h or fresh samples with regards to SPM (35.6 ± 3.9 vs 56.1 ± 6.91 and 59.3 ± 2.6), VSL (83.5 ± 4.4 vs 105.3 ± 6.5 and 110 ± 2.0) and LIN (63.9 ± 3.4 vs 75.0 ± 3.7 and 80.7 ± 2.4), respectively. A comparison between DGC-selected and non-selected samples showed a significant lower total motility [TM (%): 94.4 ± 0.8 vs 85.4 ± 1.90], VSL (176.7 ± 4.2 vs 110.0 ± 2.0) and wobble [WOB (%): 94.2 ± 0.6 vs 88.5 ± 1.5] only for fresh semen. Fluorescence analysis evidenced a decrease only in 24h cold-stored non-selected compared with fresh semen with regard to plasma membrane integrity [PMI (%): 64.8 ± 2.9 vs 80.1 ± 1.7], high mitochondrial function [HMF (%): 88.2 ± 1.6 vs 93.9 ± 1.0] and total intact plasma/intact acrosome/high mitochondrial function [IPIAHM (%): 61.8 ± 3.1vs 78.7 ± 2.0]. In contrast, no differences were observed between fresh and cold-stored DGC-selected semen. A comparison between selected and non-selected semen showed a significant increase of PMI (64.8 ± 3.14 to 89.4 ± 2.32), HMF (88.2 ± 1.26 to 96.0 ± 1.26) and IPIAHM (61.8 ± 3.14 to 87.6 ± 2.04) only for 24h. These results suggest that kinetic activity of cold-stored and DGC-selected ram spermatozoa is maintained and the selection process results in improved viability and membrane integrity. Therefore, liquid storage combined with DGC-selection might become a good alternative to fresh or frozen non-selected semen to be used for artificial insemination in sheep crossbreeding programs.