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Spindle configuration of *in vitro* matured bovine oocytes vitrified and warmed in media supplemented with a biopolymer produced by an Antarctic bacterium

N. Arcarons[1], M. Vendrell[1], M. Yeste[2], E. Mercadé[3], T. Mogas*[1]

[1]Departament de Medicina i Cirurgia Animal, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Barcelona, Spain; [2]Department de Biologia, Universitat de Girona, Girona, Girona, Spain; [3]Departament de Biologia, Sanitat i Medi Ambient, Secció de Microbiologia,Universitat de Barcelona, Barcelona, Barcelona, Spain.

Keywords: vitrification, chromosome, microtubule.

Biological molecules isolated from organisms that live under subfreezing conditions could be used to protect oocytes from cryoinjuries suffered during cryopreservation. Bacterial exopolysaccharides (EPS) constitute a common class of molecules that interact with ice in nature either by triggering ice nucleation or by inhibition of ice nucleation and growth. The aim of this work was to evaluate the spindle configuration of in vitro matured bovine oocytes vitrified/warmed in media supplemented with exopolysaccharide (M1) produced by *Pseudomonas* sp ID1 (Carrión et al., Carbohydr Polym 117:1028. 2015). After 22 h of *in vitro* maturation, a total of 546 oocytes form prepubertal (3 replicates) and 405 oocytes from adult cows (4 replicates) were vitrified/warmed in media supplemented with various concentrations of EPS M1 (0, 0.001, 0.01, 0.1 and 1 mg/ml). After warming, oocytes were allowed to recover for 2 additional hours in IVM medium. Fresh, non-vitrified oocytes were used as a control. At 24 h of IVM, oocytes from all treatments were fixed and immunostained with the Alexa-fluor 488 antibody and DAPI. Microtubule and chromosome distribution was analyzed by immunocytochemistry under a fluorescent microscope. ANOVA was performed to analyze differences in meiotic spindle configuration ($P < 0.05$). When cow oocytes were vitrified, similar percentages of normal spindle configuration were observed when compared to fresh control oocytes, except for the 0.1 mg/ml EPS M1 group that showed significantly lower rates compared to the fresh control group. Significantly higher rates of prepubertal oocytes exhibiting a normal spindle configuration were recorded in the non-vitrified group compared to all vitrified/warmed groups, regardless of the EPS M1 supplementation. However, the addition of EPS M1 to the vitrification/warming media decreased the ratio of decondensation or absence of chromosomes and microtubules in prepubertal oocytes. Although percentages of normal spindle configuration after vitrification were lower for prepubertal than for cow oocytes, no significant differences were observed when oocytes were vitrified with 0.001, 0.1 and 1 mg/ml EPS M1. In conclusion, supplementation with EPS M1 concentrations during vitrification and warming did not induce adverse changes in the spindle of bovine oocytes, regardless of the concentration used. Although a more severe damage on spindle configuration could be observed after vitrification of prepubertal oocytes, EPS supplementation during vitrification and warming seems to have a greater benefit during vitrification of prepubertal than adult bovine oocytes. Further experiments are required to investigate if *in vitro*-matured oocytes vitrified/warmed in presence EPS M1 can improve their development competence after being vitrified/warmed. This study was supported by the Spanish Ministry of Science and Innovation (Project AGL2016-79802-P and grant CTQ2014-59632-R).

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Comparison of lipid profiles and gene expression in granulosa and cumulus cells in bovine

P. Bertevello*[1], C. Banliat[1], A.P. Teixeira-Gomes[1], S. Elis[1], V. Maillard[1], A.V. Carvalho[2], V. Duranthon[2], V. Labas[1], S. Uzbekova[1]

[1]INRA, UMR Physiologie de la Reproduction et des Comportements, Nouzilly, 37380, France; [2]INRA, UMR Biologie du Développement et de la Reproduction, Jouy en Josas, 78352, France.

Keywords: *cumulus, granulosa, lipid metabolism.*

Cumulus cells (CC) derive from granulosa cells (GC) during follicular growth and antrum formation and are coupled with an oocyte. Fatty acid (FA) synthesis and oxidation in GC impact cell proliferation and steroidogenesis (Elis et al, 2015 Theriogenology. 2015, 83(5):840-53) whereas in CC these processes are crucial for oocyte maturation (Sanchez-Lazo et al, 2014, Mol Endocrinol. 2014 28(9):1502-21). Both GC and CC contribute to oogenesis and reflect oocyte quality. The objective of our study was to compare intracellular lipids and lipid related transcripts between these compartments. Lipid profiles obtained using MALDI-TOF mass spectrometry were compared between GC from individual follicles (n=12) and pools of CC (n=12) aspirated from 4-5 mm follicles of slaughtered cows. Freshly isolated cells were analysed using UltrafleXtreme MALDI-TOF/TOF instrument (Bruker) in positive (+) and negative (-) reflector mode, with 2,5-dihydroxyacetophenone matrix. Peaks were detected in 100 -1000 m/z range and values of the normalized peak heights (NPH) were quantified using Progenesis MALDI™ (Nonlinear Dynamics). Student's t-test was applied to NPH values for hunting lipid content variations between GC and CC. Peaks were annotated using MS/MS fragmentation confronted to lipid databases. Lipid fingerprints from CC and GC gathered 462 peaks in (+) and 486 peaks in (-) modes, with coefficients of variation = 27% for CC and 18% for GC. 143 species were significantly upregulated in CC ($P<0.01$, fold change >2.0). Among them, 2 lyso-phosphatidylcholines (LPC 20:4 and 20:3) 12 phosphatidylcholines (PC), and 12 sphingomyelins (SM) were identified. Among 44 molecular forms which were more abundant in GC, we identified LPC (14:0), 4 PC, ceramide (22:1), SM (15:1), phosphatidylethanolamines (28:0 and 38:7) and phosphatidylserine (29:0). 4 peaks were annotated as triglycerides. Gene expression in pools of CC (n=4) and GC (n=4) was analyzed using a customized 60K bovine microarray (Agilent technology, 61326 probes). Differential analysis revealed 2009 differentially expressed genes (DEG) which were up-regulated in CC and 694 in GC ($P<0.05$, Benjamini-Hochberg correction). DEG showed significant enrichment in the pathways related to carbon metabolism, glycolysis /gluconeogenesis, ATP-binding cassette transporters, amino acid and O-glycan biosynthesis, thyroid hormone, PI3K-Akt signaling, p53 and PPAR signaling pathway (corrected $P<0.05$). Among the DEG related to lipid metabolism and regulated by PPARs, genes *ACOX2, LPL, SCD, PPARG, FABP3, FADS2, ACADL, SLC27A2* were up-regulated in CC and *CPT1A, CPT1B, SCD5, PLTP* were more expressed in GC. Ten sphingolipid metabolism genes were over-expressed in CC. In conclusion, numerous genes related to lipid metabolism were differently expressed in CC and GC. This corroborates differences in GC and CC lipids and may reflect different involvement of GC and CC in glyceroneogenesis, lipogenesis, oxidation and steroid production.
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Reproductive Pixel Grey-Intensity Score with Image-J and Freezability in Angora Bucks

B. Cil*[1], K. Tekin[1], C. Stelletta[2,1], A. Daskin[1]

[1]Department of Reproduction and Artificial Insemination, Ankara University, Faculty of Veterinary Medicine, Ankara, Turkey;
[2]Department of Animal Medicine, Production and Health, University of Padova, Padova, Italy.

Keywords: Angora goat, pixel gray intensity, ultrasound.

We aim to evaluate male reproductive tract ultrasonography and its relation with sperm quality and freezability in Angora bucks. A total of five Angora bucks were selected with age range (2-3 years). The reproductive monitoring of testicular and accessory glands was assessed with ultrasound (US) (Esaote® MyLab One, Italy). Scrotal circumference, total testicular volume, epididymal area, vesicular gland area and bulbourethral gland area were calculated. Semen collection through the Trans-rectal massage (TM) and collection time were recorded. Semen is frozen with Tris egg-yolk diluent and post-thaw Delta (Δ) motility, Δ progressivity and kinetic parameters with The Hamilton-Thorn computer-aided semen analyzer, version 10 Ivos (HTR analyzer, Hamilton-Thorn Research, Beverly, MA, USA), validated for buck semen analysis. Mean differences between PGI values and post-thaw motility were evaluated by paired Student's t-test. All the US images collected were analyzed for their pixel gray intensities (PGI) using Image-J software and classified depending on the difference between the average group value. Regression analysis was carried out among all the parameters collected. Post-thaw Delta (Δ) Medians of motility, progressivity, VAP, VSL, VCL, Lateral Amplitude, Straightness and Linearity were -23.22, -20.22, -0.04, -0.92, -10.21, 0.13, 1.13, and 0.36 respectively. High levels of relationship ($P<0.05$) were identified among ejaculate volume, PGI and area selected of the epididymis. Besides, a high relationship was identified between Total testicular volume and pixel gray intensity. Delta score and collection time was also positively correlated. We concluded that extreme PGI levels seem to be related to the worst sperm quality in terms of motility. Ultrasound monitoring represents an innovative technology, which may give a high impulse to the field application of the BSE in small ruminants. PGI of the reproductive tract result as useful diagnostic tools for sperm quality assessment and genetic material use.

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Cell-signalling metabolites predominate among small molecules differently released by male and female bovine embryos cultured *in vitro*

E. Gomez*[1], D. Martin[1], S. Carrocera[1], P. Herrero[2], N. Canela[2], M. Muñoz[1]

[1]SERIDA, Gijon, Asturias, Spain; [2]Universitat Rovira i Virgili, Reus, Cataluña, Spain.

Keywords: bovine, embryo, sex.

The cow uterus recognizes embryonic sex, and male and female early bovine embryos show dimorphic transcription that impacts metabolism. Most metabolites are small molecules that may exert a role within early embryo maternal interactions. Individual release of metabolites was examined in a 24h single culture medium from Day-6 male and female morulae that developed to Day-7 expanded blastocysts. Embryos were produced in vitro from slaughterhouse oocytes, fertilized with a single bull and cultured in SOFaaci+6g/L BSA. Prior to metabolomics analysis, embryos had their sex identified (amelogenin gene amplification). Embryos (N=10 males and N=10 females) and N=6 blank samples (i.e. SOFaaci+6g/L BSA incubated with no embryos) were collected from 3 replicates. Metabolome was analysed by UHPLC-TOF-MS in spent culture medium as described allowing identification of 5 sex biomarkers (Gómez et al, J Chromatogr A 2016; 1474:138–144). The remaining output data were submitted to Principal Component Analysis (PCA) to detect outliers, Kolmogorov-Smirnov test to evaluate normality and Levene's test to assess the equality of the variances. Thereafter, analysis of variance by one-way ANOVA was performed to detect the different peak area averages ($P<0.05$). We found 1,720 metabolite signals showed significant differences between male and female embryos. Potential metabolites were tentatively identified by matching the m/z to those published in the Human Metabolome Database within a mass accuracy window of 10 ppm. In addition, Molecular Formula Generator algorithm of MassHunter software (Agilent) was used to support the tentative identification considering their isotopic distribution. N=13 metabolites were differentially identified. LysoPC(15:0) was the only metabolite found at higher concentration in females (fold change [FC] male to female = 0.766). FC of metabolites more abundant in male (12) varied from 1.069 to 1.604. Chemical taxonomy grouped metabolites as amino-acids and related compounds (DL-2 aminooctanoic acid, arginine, 5-hydroxy-L-tryptophan, and palmitoylglycine); lipids (2-hexenoylcarnitine; Lauroyl diethanolamide; 5,6 dihydroxyprostaglandin F1a; LysoPC(15:0); DG(14:0/14:1(9Z)/0:0) and triterpenoid); endogenous amine ((S)-N-Methylsalsolinol/(R)-N-Methylsalsolinol); n-acyl-alpha-hexosamine (N-acetyl-alpha-D-galactosamine 1-phosphate); and dUMP, a product of pyrimidine metabolism. Among the compounds originally contained in CM, female embryos significantly depleted more arginine than males and blank controls ($P<0.001$). Male and female embryos induce different concentrations of metabolites with potential signalling effects that may facilitate sex recognition in the uterus. The increased abundance of metabolites released from males is consistent with the higher metabolic activity attributed to such blastocysts. MINECO-project AGL2016-78597. Principado de Asturias, Plan de Ciencia, Tecnología e Innovación 2013-2017 (GRUPIN 14-114) and FEDER. The authors are members of the COST Action 16119, In vitro 3-D total cell guidance and fitness (Cellfit).

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In vitro assessment of acrosomal status of boar sperm bound to beads conjugated to ZP proteins

J.G. Hamze*[1], R. López-Úbeda[1,3], A. Canha-Gouveia[2], M. Jimenez-Movilla[1], R. Romar[2]

[1]Departamento de Biología Celular e Histología, Facultad de Medicina, Universidad de Murcia. IMIB., Murcia, España; [2]Departamento de Fisiología, Facultad de Veterinaria, 30071 Campus Espinardo,, Murcia, España; [3]FFIS (Fundación para la Formación e Investigación Sanitarias de la Región de Murcia)., Murcia, España.

Keywords: gamete interaction, ZP conjugated-beads, 3D model.

The oocyte's zona pellucida (ZP), composed by 3 or 4 glycoproteins, mediates the interaction with sperm. An *in vitro* 3D model mimicking the oocyte's shape based on studies showing the relevance of the processing of ZP2 at N terminal position in sperm-oocyte interaction (LADEN) (Avella, Sci Transl Med 8:336ra60, 2016) is being used to study the molecular mechanisms involved on gamete interaction in pigs (Hamze, Animal Reprod 13: 647, 2016). The model consists of magnetic beads (His Mag Sepharose™ Excel) conjugated with porcine ZP2, ZP3 and ZP4 recombinant proteins. The objective was to study the binding kinetics and acrosomal status of boar spermatozoa bound to beads conjugated with ZP2, ZP3 or ZP4. ZP2 and ZP4 models were produced as previously described (Hamze, Animal Reprod 13: 647, 2016) and ZP3 was identified by electrophoresis and western blot with anti-ZPC polyclonal antibody showing a molecular weight of 55kDa. Once produced, 45-50 ZP proteins conjugated-beads were incubated in TALP medium (500 ul) with 200.000 boar spermatozoa/ml for 30, 60 and 120 min. At each time, an aliquot of beads was washed (PBS), fixed (0.5% glutaraldehyde, 30 min) and stained for 15 min with bisbenzimide (0.01mM) and Peanut agglutinin (PNA, 4µg/µL) to evaluate the number and acrosomal status of sperm bound to the beads . Three replicates with a blind analysis were done. We calculated the rate of beads with at least one sperm bound (BSB), the mean number of sperm per bead (S/B) and the acrosomal reaction of bound sperm. Results were analysed by one-way ANOVA considering statistical differences when *P*-value <0.05. After 30 min of coincubation, the BSB was higher for ZP3 and ZP4 (71.54 ± 3.67%, n=158 and 75.56 ± 3.53%, n=155) than for ZP2 (56.52 ± 4.01%, n= 154) and the S/B was higher for ZP4 (3.87 ± 0.31) than ZP2 (2.58 ± 0.18) and ZP3 (2.69 ± 0.17). No differences were observed at 60 min for BSB but S/B was higher for ZP2 (6.66 ± 0.43) than ZP3 (4.87 ± 0.31) and ZP4 (4.23 ±0.24). Finally, at 120 min both BSB and S/B were higher for ZP2 (93.5 ± 2.0%, 9.00 ± 0.45) and ZP3 (93.6 ± 2.0%, 8.54 ± 0.49) than ZP4 (81.0 ± 3.2%, 6.68 ±0.61). After 30 min of incubation the ZP2 model induced a higher acrosome reaction since 77.50 ± 3.76% of the bound sperm had reacted whereas for ZP3 and ZP4 models rates were 69.84 ± 3.84 and 65.04 ± 3.43, respectively. No differences were found at 60 and 120 min probably due to the high capacitation ability of TALP medium. In conclusion, ZP2 conjugated beads bound a higher number of reacted spermatozoa at 30 min. Regarding binding kinetics, differences between groups were observed through time, being ZP2 and ZP3 conjugated beads the models with a higher S/B at 120 min. The full development of this 3D model will permit in the future a better and deeper understanding of gametes interaction in pigs and the reduction of female gametes in gametes interaction studies. Supported by MINECO and FEDER (AGL2015-70159-P) and Fundación Seneca-Agencia de Ciencia y Tecnología de la Región de Murcia (18931/JLI/13), "Jóvenes Líderes en Investigación".

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Efficiency of Preimplantation Genetic Diagnosis (PGD) of bovine IVP embryos using blastocoele fluid or embryonic cells

C. Herrera*[1,2], S. Wyck[1], L. Bittner[1], F. Janett[1], H. Bollwein[1]

[1]Clinic of Reproductive Medicine, Vetsuisse Faulty, University of Zurich, Zurich, Switzerland; [2]Center for Clinical Studies, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland.

Keywords: embryo sexing, PGD, blastocoele fluid.

Preimplantation Genetic Diagnosis (PGD) involves collecting a few cells from a preimplantation embryo, which will then be used for genetic testing. Palini et al. were able to diagnose the sex human embryos using only the blastocoele fluid as a source of DNA [Palini, Reprod Biomed Online, 26(6):603-10, 2013]. Recently, we demonstrated that the DNA in the blastocoele fluid of bovine IVP embryos can be amplified by PCR to diagnose the sex of the embryos [Herrera, Proceedings in 42° Veterinär-Humanmedizinische Gemeinschaftstagung, Abstract 51, 2017]. The aim of our work was to compare the efficiency of PGD of bovine IVP embryos using blastocoele fluid or embryonic cells as a source of DNA for sexing the embryos by PCR. Bovine embryos were produced in vitro and all expanded blastocysts were randomly assigned to one of three experimental groups: 1) Collapsed Embryos (CE): blastocoele fluid was collected from blastocysts, 2) Biopsied Embryos (BE): 1 to 5 cells were collected form blastocysts by aspiration and 3) Intact Embryos (IE): blastocysts were left intact. In 1) and 2) blastocyst stage embryos were placed under an inverted microscope equipped with a micromanipulation system for the collection of blastocoele fluid or embryonic cells, as decribed previously for equine embryos [Herrera, Theriogenology 81(5):758-63, 2014; Herrera Theriogenology 83(3):415-20, 2015]. Collapsed, biopsied or intact blastocysts were vitrified and warmed using the vitrification method described by Vajta et al. (1999), except a hemistraw instead of an Open Pulled Straw (OPS) was used as a carrier. After warming, embryos were cultured in vitro and observed for 48 h to detect reexpansion and hatching. The DNA from the blastocoele fluid or from the embryonic cells was amplified by PCR as decribed previously [Herrera, Proceedings in 42° Veterinär - Humanmedizinische Gemeinschaftstagung, Abstract 51, 2017]. The survival rates after warming and in vitro culture for 48 h and the efficiency of amplification after PCR were compared by ANOVA and Fisher's exact test between the experimental groups. The post-warming survival rates of blastocysts did not differ significantly between CE, BE or IE (93.1%, 96.8% and 95.6% respectively) ($P>0.05$). The hatching rates after warming and 48 h of IVC, did not differ between BE or IE (75% vs. 47.8%), was significantly higher for CE when compared to IE (79.3% vs. 47.8%) and did not differ between CE and BE. The amplification rates after PCR was significantly higher for blastocoele fluid samples (41/41, 100%) that for biopsied cells (30/34, 80.3%) ($P<0.05$). The present results demonstrate that blastocoele fluid can be collected from IVP blastocysts and used as a source of DNA for PCR, without impairing the viability of the embryo. In our hands, the use of blastocoele fluid was more efficient than cells after PCR.

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Bovine embryo lipid metabolism is affected by perfluorononanoic acid (PFNA) exposure during oocyte maturation *in vitro*

J. Kjellgren[1], I. Hallberg[1,3], S. Örn[2,3], S. Persson[1,3], Y.C.B. Sjunnesson*[1,3]

[1]Swedish University of Agricultural Sciences (SLU), Department of Clinical Sciences, Division of Reproduction, Uppsala, Sweden; [2]Swedish University of Agricultural Sciences (SLU), Department of Biomedical Sciences and Veterinary Public Health, Section of Environmental Toxicology, Uppsala, Sweden; [3]Centre for Reproductive Biology in Uppsala, University of Agricultural Sciences (SLU) and Uppsala University, Uppsala, Sweden.

Keywords: cow, reproductive toxicology, *in vitro* embryo production.

The final maturation of the oocyte, fertilization and the early embryo development are sensitive processes that can be affected by chemicals. In reproductive toxicology studies, bovine *in vitro* production (IVP) of embryos provides a controlled setting where testing of chemicals is possible without the use of laboratory animals. Bovine IVP is a good model for humans. PFNA is used in consumer products such as water and stain/oil repellent products. The substance is highly persistent and found in both wildlife and nature, but also in human follicle fluid (0.2-2.1 ng/ml (Petro et al., Sci Total Environ 496, 282–288, 2014)). Studies regarding PFNAs potential toxicological effects, especially developmental toxicity, are limited. The aim of this study was to explore the bovine IVP model (Abraham et al., Acta Vet Scand 54:36, 2012) and examine effects of PFNA exposure on oocytes during *in vitro* maturation by evaluation of blastocyst mitochondrial and lipid status since these are suspected to be involved in the mode of action via oxidative stress and peroxisome proliferation-activated receptor α. Abattoir-derived bovine ovaries were used to collect cumulus oocyte complexes (n = 440). The oocytes were matured *in vitro* under PFNA exposure (100 ng/ml to include a safety margin) or non-exposed controls. *In vitro* fertilization and culture were done according to standard protocols. Embryo development was assessed by cleavage rate and blastocyst development and morphology. Day 8 blastocysts were stained for visualization of active mitochondria (MitoTracker® Orange CMTMRos, ThermoFisher Scientific, Waltham, USA) and fixed in paraformaldehyde. Additional staining was done with nuclear stain (Deep Red Anthraquinone 5, BioNordika, Stockholm, Sweden) and neutral lipid stain (HCS LipidTOX™ Green Neutral Lipid Stain, ThermoFisher Scientific, Waltham, USA). For analysis of the embryos confocal laser scanning microscope was used (LSM 510, Carl Zeiss Microscopy GmbH, Oberkochen, Germany). The embryos were manually evaluated. Mitochondrial staining was evaluated with regards to distribution (even distribution to uneven distribution with areas devoid of mitochondria or distinct aggregations of mitochondria). Neutral lipid staining was evaluated with regards to dominating size of lipid droplets. Statistical analyses were performed by linear mixed models and generalized linear mixed models, with replicate as random factor and observations on day 7 and 8 as repeated measures. There was no significant difference ($P>0.05$) between treated and control group regarding, cleavage rate, blastocyst development day 7 and 8, quality grade of blastocysts, stage of blastocysts, number of nuclei or mitochondrial distribution scoring. However, there was a significant difference in distribution of lipid droplet size where the treated group had an increased amount of large lipid droplets ($P = 0.048$). To conclude, the bovine IVP model suggests a disturbance in lipid metabolism but the exact working mechanism of PFNA must be further explored. Funded by Formas 2015-476.

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Bull spermatozoa have better membrane integrity and mitochondrial membrane potential when cryopreserved with a liposome-based extender

I. Lima-Verde*[1], A. Johannisson[2], T. Ntallaris[2], A. Edman[3], J.M. Morrell[2]

[1]Tiradentes University, Technology and Research Institute, Aracaju, Sergipe, Brazil; [2]Clinical Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden; [3]Viking Genetics, Skara, Sweden.

Keywords: semen, single layer centrifugation, liposome-based extender.

Functional, robust spermatozoa are needed for assisted reproduction biotechnologies. Good quality spermatozoa can be separated from the rest of the ejaculate using colloid centrifugation, especially Single Layer Centrifugation (SLC) (Morrell et al., Animal Reproduction 13, 340-345; 2016). To avoid including material of animal origin, such as egg yolk, commercial extenders have been developed containing soy lecithin or liposomes. The aim of this study was to evaluate the effects of SLC on bull sperm quality when spermatozoa were frozen in these extenders. Semen was collected from 12 bulls at a commercial bull station (Viking Genetics, Skara, Sweden). Immediately after collection, each semen sample was split into control and SLC samples. Controls were extended to provide a sperm concentration of 69×10^6 spermatozoa/mL, in AndroMed® (Minitube, Tiefenbach, Germany - soy lecithin based extender - control A) or OptiXcell® (IMV Technologies, L´Aigle, France - liposomes based extender - control O). SLC samples were extended to 50×10^6 /mL in Tris-egg yolk prior to centrifugation through the colloid Bovicoll; after SLC the sperm pellet was resuspended in AndroMed® (SLC A) or OptiXcell® (SLC O) to the same concentration as controls. All samples were frozen in 0.25 mL plastic straws. After 3-10 days storage in liquid nitrogen, the straws were thawed at 37°C for 12 s for sperm quality evaluation. Analyses of membrane integrity (MI) and mitochondrial membrane potential (MMP) were made by flow cytometer. Means were analysed using the proc mixed procedure for linear mixed models. Correlations were calculated using Pearson´s correlation test; Scheffe's adjustment was used for multiple-post ANOVA comparisons. Results are presented as LSMeans ± Standard error of means (SEM); the differences were considered significant at $P<0.05$. The samples cryopreserved with OptiXcell® showed better MI and MMP ($P<0.05$) than those cryopreserved with AndroMed®, both in control groups (control O versus control A, MI: 48.5+3.2 vs. 38.8+3.2; MMP: 55.8+3.0 vs 34.1+3.0) and SLC groups (SLC O versus SLC A, MI: 45.5+3.2 vs. 30.8+3.2; MMP: 66.4+3.0 vs. 41.4+3.0). Within extender, no differences were observed between control and SLC for MI ($P>0.05$) (control A versus SLC A, 38.8+3.2 vs. 30.8+3.2; control O versus SLC O: 48.5+3.2 vs. 55.8+3.0) or MMP (control A versus SLC A: 34.1+3.0 vs. 41.4+3.0; control O versus SLC O: 55.8+3.0 vs. 66.4+3.0). In conclusion, bull spermatozoa, selected or not by SLC, have increased MI and MMP when the liposome-based extender was used. Acknowledgement: I Lima-Verde received a fellowship from Brazilian Council of Research (CNPq-Brazil). The project was funded by a project grant from the Swedish Farmers´ Foundation (SLF; H13300339) awarded to JM Morrell and A Johannisson.

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Comparison of the survival rates of ovarian tissue after slow freezing and vitrification by assessing histological structure and estradiol production during in-vitro culture

D. Nikiforov*, V. Russo, D. Nardinocchi, N. Bernabò, M. Mattioli, B. Barboni

University of Teramo, Teramo, Abruzzo, Italy.

Keywords: cryopreservation, ovarian tissue.

Our study was designed to identify a cryopreservation technique, which ensures better surviving of ovarian tissue. The aim is to compare effectiveness of slow freezing (SF) and vitrification (VIT), followed by in-vitro culture and histological analysis. All reagents were purchased from Sigma Aldrich SRL (Milan, Italy), unless other specified. Cortical tissue was isolated from pubertal ovine ovaries, transported from the local slaughterhouse. Slivers (1*5*5 mm) were randomly allocated into six groups (n=5 in each): 1-non frozen control, 2-SF protocol, 3-VIT protocol, 4-non frozen control for in-vitro culture (IVC), 5-SF protocol for IVC, 6-VIT protocol for IVC. The cryoprotectants used in SF protocol were 1.5M ethylene glycol (EG) and 0.1M sucrose (SUC). Vials with samples were thawed in a water bath at 37ºC and then washed in phosphate buffered saline containing 0.75M EG and 0.25M SUC. In VIT protocol cryoprotectants were 2.5M dimethyl sulfoxide, 2.5M EG and 0.5M SUC. Warming performedn at 37ºC in McCoy's 5a medium contained 0.5M SUC and then washed in the medium with 0.25M SUC. For the histological analysis pieces of tissue were fixed in 4% paraformaldehyde, then dehydrated in series of ethanol and embedded in paraffin. The samples were sectioned (5 μm) and stained with hematoxylin and eosin. Follicles in the tissue were assessed by criteria established in our laboratory (Martelli et al., J Mol Endocrinol, 2006) and classified into three quality groups: intact, partially damaged and degenerated. Cortical strips were cultured in McCoy's 5a medium for 6 days at 37ºC and 5% CO2 with medium changed every 2 days. Then culture medium was analysed for the content of estradiol (E2) by ELISA assay (DRG, Marburg, Germany). The proportion of normal follicles showed significant difference between SF (total number of follicles counted=177) and VIT groups (total number of follicles=223): 27,96% vs 19,36% (P<0,001, χ^2 test). After the in-vitro culture, 84 and 69 follicles in total were counted for the SF and VIT groups, respectively. In this case, a higher percentage of intact follicles after slow freezing also has been shown: 21,87% vs 16,52% (0<0,001, χ^2 test). The mean E2 concentrations for days 1,3 and 6 of in-vitro culture after SF protocol were 3,1 pg/ml; 11,4 pg/ml and 12,1 pg/ml, which were 20% lower, than values for non-frozen control (3,7 pg/ml; 13,6 pg/ml and 14,6 pg/ml). However, the difference of E2 concentration from the non-frozen control was even greater for the VIT group, where the values were more than 50% lower: 1,8 pg/ml; 5,9 pg/ml and 7,6 pg/ml. An increase of E2 concentrations during the in-vitro culture was observed, which proved tissue recovering after cryopreservation. Slow freezing is ensuring better morphological structure of ovarian cortex than vitrification. More specifically, a higher number of morphologically healthy follicles could be seen and a better production of estradiol during in-vitro culture of ovarian slivers was present.

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Annual control chart of bull semen freezability

D. Sahin*[1], I. Bastan[1], B. Çil[2], K. Tekin[2], C. Stelletta[3], M. Satilmis[1], E. Akcay[2], A. Daskin[2]

[1]Livestock Central Research Institute, Ankara, Turkey; [2]Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey; [3]Department of Animal Medicine, Production and Health, University of Padova, Padova, Italy.

Keywords: freezability, bull semen, CASA.

Optimization of the center bull production is depending on the application of a fine control chart creation. The use of CASA system improved the precision and accuracy on the early identification of production problems. Aim of the present work was the modelling of a control chart that considered the monthly variations during a whole year in terms of semen freezability and number of straws produced. A total of 536 ejaculates were immediately evaluated, diluted with Andromed® and frozen using an automatized freezer (Digitcoo5300 ZV, IMV). Thereafter the straws were plugged in liquid nitrogen. Fresh and post-thawed motilities where evaluated. Computer assisted sperm analysis (CASA) parameters were analyzed considering the monthly variations of individual kinetic characteristics as average pathway velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL) and hyperactive % (HYP). The variations were scored in terms of number of SD from the average value for each parameter. Delta motility (difference between post-thawed and fresh semen motility) was higher during the summer and autumn period and in Holstein bulls (up to -30%). Collected and elaborated data were analyzed through ANOVA for repeated measures using month and season as independent variables while breed, semen quality parameters and number of straws as dependent variables. The number of produced straws were significantly changed between the spring and the summer months indicating the lower resistance of the bulls to the hot environment. CASA parameters as VAP, VCL, VSL and HYP reinforce the indication that heat stress can influence up to three months the freezability of bull semen.

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Effect of conceptus size on embryo-maternal communication during early pregnancy in cattle

J.M. Sánchez*[1], D.J. Mathew[1], C. Passaro[1], G. Charpigny[2], S. Behura[3], S.T. Butler[4], T.E. Spencer[3], P. Lonergan[1]

[1]School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland; [2]INRA, Biologie du Développement et Reproduction, 78350, Jouy en Josas, France; [3]Division of Animal Sciences, University of Missouri, Columbia, Missouri, USA; [4]Animal Research Department, Animal and Grassland Research and Innovation Centre, Teagasc, Moorepark, Fermoy, County Cork, Ireland.

Keywords: uterine-explant, conceptus, transcriptome.

Conceptus elongation is an essential prerequisite to maternal recognition of pregnancy and implantation in cattle. During elongation, the trophoblast cells secrete interferon-tau (IFNT), which prevents the upregulation of oxytocin receptors in the endometrium required for prostaglandin-induced luteolysis. Large variation exists in the length of conceptuses recovered on the same day of gestation, which may reflect an inherent lack of developmental competency. For example, larger conceptuses produce more IFNT, but the underlying factors that regulate conceptus-maternal crosstalk between advanced (large) or retarded (small) conceptuses and the endometrium are unknown. Thus, the aim of this study was to interrogate the response of the endometrium to Day 15 conceptuses of different sizes by examining the global transcriptome profiles of uterine explants exposed to large vs small conceptuses. First, 10 grade 1 *in vitro* produced blastocysts were transferred into synchronized recipient heifers on Day 7 for further development. The resulting conceptuses were recovered on Day 15 by *post-mortem* uterine flushing. Seven large (mean length ± SEM 25.4 ± 5.7 mm) and six small conceptuses (1.8 ± 0.3 mm) were individually placed on top of endometrial explants that had been collected from uteri during the late luteal phase of the estrous cycle, and co-cultured for 6 h in one mL of RPMI media. Additional explants were cultured with media containing 100 ng/mL of recombinant ovine IFNT (IFNT; n=6) to identify endometrial responses dependent and independent of IFNT or in media alone (Control; n=6). Total RNA was isolated from explant cultures and analysed by RNA-Seq. Exposure of endometrium to IFNT, a large conceptus or a small conceptus altered ($P < 0.05$) expression of 491, 498 and 230 transcripts, respectively, compared with control endometrium. Further, 223 differentially expressed transcripts were common between conceptus-treated and IFNT-treated explants, and classical interferon-stimulated genes (e.g., RSAD2 and ISG15) were amongst the most upregulated transcripts compared to control endometrium. In addition, 369 transcripts were uniquely altered in explants exposed to large conceptuses and IFNT. Of these transcripts, 101 and 100 were specific to large conceptuses and IFNT-treated endometrium, respectively, while 168 were common to both groups. Only 6 of 108 conceptus-induced differentially expressed genes were shared between small- and large conceptuses. Interestingly, 101 transcripts were exclusively regulated by large conceptuses; of these, *PCSK1*, *TNNI3K*, *MPV17L*, and *IL17* were the most upregulated and *TEPP*, *CACNA1L*, *AQP1*, and *HIP1* the most downregulated. This study provides new knowledge of differences in gene expression in endometrial tissue induced by large and small conceptuses. The results provide a better understanding of the underlying molecular pathways involved in embryo survival and maternal recognition of pregnancy in cattle. Funded by Irish Department of Agriculture, Food and The Marine through the Research Stimulus Fund (Grant number: 13/S/528).

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Development competence of bovine oocytes selected by brilliant cresyl blue before vitrification

A. Serikov*[1], T. Kuzmina[2], Y. Ussenbekov[3], K. Koibagarov[3]

[1]Ulsan National Institute of Science and Technology, Ulsan, Republic of Korea; [2]Russian Research Institute of Farm Animal Genetics & Breeding, St.Petersburg-Pushkin, Russia; [3]Kazakh National Agrarian University, Almaty, Kazakhstan.

Keywords: oocyte, vitrification, cow.

Numerous factors influence *oocyte cryoresistance*, including diameter of follicle, meiosis stage, functional status of oocytes etc. (Papis K., Slovak J. Anim. Sci., 48, 2015 (4): 163-171). It was shown that bovine oocytes selected by brilliant cresyl blue (BCB) had different developmental competence. Native BCB^+ oocytes (oocytes that have finished growth phase in vivo) had significantly higher development competence than oocytes that have not finished growth phase in vivo (BCB^- oocytes, Heleil B. et al., J. Reprod. & Infertility 1 (1):01-07, 2010).In previous studies we demonstrated that pretreatment of bovine oocytes with follicular fluid from follicle 3 mm before vitrification improves nuclear and cytoplasmic maturation and development of vitrified bovine oocytes (Kuzmina T.I. et al., *Reprod Biomed online,* 20(3): S38–S39, 2010). The aim of the present study was to evaluate the development competence of devitrified BCB^+ and BCB^- oocytes. Before vitrification cumulus oocyte complexes (COCs) were incubated in BCB solution (26 μM) for 90 min. Oocytes were divided into BCB^- (colorless cytoplasm) and BCB^+ (colored) and then COCs were incubated 40 min in follicle fluid (d of follicles ≤ 3 mm). Vitrification was performed by equilibration of oocytes in CPA (Cryoprotective Additive) - 1: 0.7 M dimethylsulphoxide (Me2SO) + 0.9 M ethylene glycol (EG) (30 sec); CPA-2: 1.4 M Me2SO + 1.8 M EG (30 sec); CPA-3: 2.8 M Me2SO + 3.6 M EG + 0.65 M trehalose (20 sec) and loading into straws. After thawing COCs washed by step-wise dilution in 0.25 M,0.19 M and 0.125 M trehalose in TCM-199 and finally in TCM-199 alone. COCs were cultured in TCM 199 + 10% (v/v) heat-treated FCS + 50 ng/ml PRL with 10^6/ml granulosa cells. COCs were cultured in this medium 15 h. Then medium were supplemented by 10 IU/ml hCG. The time of cultivation for BCB^+ and BCB^- oocytes were 24 h. After IVM oocytes were fertilized in vitro and embryos were cultured by standard protocols. Cleavage and development rates were examined on days 2 and 7 after fertilization, respectively. All chemicals used in this study were purchased from Sigma – Aldrich (Moscow, Russia). A total of 604 COCs were vitrified**,** 399 COCs were treated by BCB. Cleavage was significantly higher in BCB^+ oocyte in compared to BCB^- oocytes [51% (103/201) vs. 31 %(61/198), respectively, $P<0.05$, χ^2 test]. Blastocyst development rate was significantly higher in BCB^+ vs. BCB- oocytes [9% (18/201) vs. 1% (2/198), respectively, $P<0.05$, χ^2 test]. In the control group (oocytes have not treated by BCB) the cleavage and blastocyst development rates were significantly low in comparison to the BCB^+oocytes [39% (80/205) vs 51% (103/201) and 3(6/205) vs 9(18/201), respectively, $P<0.05$, χ^2 test]. We have not find differences in cleavage and blastocyst development rate between control group of oocytes and group of BCB^- oocytes. In conclusion, BCB test is an effective method for selection of more competent bovine GV- oocytes for vitrification.

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Simplifying the oviductal cell adhesion test for bovine sperm quality assessment

C. Stelletta*[1,2], K. Tekin[2], J. Vencato[1], G. Gollin[1], G. Bertoli[3], L. Cestaro[3], M.B. Tirpan[2], A. Daskin[2], S. Romagnoli[1]

[1]Department of Animal Medicine, Production and Health. University of Padova, Legnaro, Padova, Italy; [2]Department of Animal Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey; [3]Intermizoo S.p.a., Padova, Italy.

Keywords: Oviductal adhesion test, sperm quality, endangered breed.

Classic procedures for semen evaluation in breeding soundness evaluation require time and high cost. The evaluation techniques with CASA has limits due to the lack of tools standardization in different laboratories and to data analysis methods. Recent studies cast doubts on the actual predictive ability of this method regarding semen fertility at field level. Therefore, many studies have focused on finding a simple and objective test which can give the maximum correlation between in vitro and in vivo results. In this study, we aim to develop a repeatable protocol of sperm adhesion test using oviductal explant (AOC) and comparing the results with CASA parameters and field fertility (ERCR). In this test, the interaction between spermatozoa and oviduct was assessed by incubation in co-culture oviductal explants, in order to calculate the number of spermatozoa adhered per unit area or adhesion index (AI) and create a correlation between this index and field fertility. Oviductal explant cells on the glass slide were exposed to 5uL of diluted semen, containing approximately 35000 motile sperms. Slides were incubated for the 20 mins. Subsequently, reading of the slide by means of optical microscope (Olympus CX41) and without any staining with magnification of 400 X was performed. A mobile field in which the explant cells of oviductal rectilinearly ran through the major diameter of the field was considered and only motile sperm still adhering to one side of explant cell were counted. Three optic fields (OF) for each slide and the average of the three counts are evaluated. Class 1 (ERCR> 1.00): high fertility; Class 2 (ERCR -1 / + 1): medium fertility; Class 3 (ERCR <1.00): low fertility. Five Frisona breed bulls within each different class have been selected randomly and for each bull three doses of semen belonging to the production lots that have contributed to the definition ERCR were considered. Three doses of semen were considered and for each sample were counted sperm immobilized on three microscopic fields. AI was different ($P<0.05$) among the ERCRs with 26.22±2.34, 17.9±1.44 and 12.64±1.68 sperms/OF for Class 1, Class 2 and Class 3 respectively. After the developmental phase, AOC was applied to a group of endangered Burlina breed bulls (N:8) where the effective field results are difficult to obtain due to the small size of population. Burlina bulls resulted with an AI lower then Class 1 with 15.21±1.14 sperms/OF. AOC test provides a prediction on bull semen fertility. Counting the sperms adhered in three microscopic fields after a co-incubation in PBS can give useful information on the field fertility level. AOC gives additional information to the standard of semen evaluation methods applied to endangered breeds.

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Effects of polyvinyl alcohol on fresh and post-thawed physiological motion characteristics of Angora goat semen

K. Tekin*[1], C. Beste[1], D. Sahin[3], I. Bastan[3], C. Stelletta[2], A. Daskin[1]

[1]Department of Reproduction and Artificial Insemination, Ankara University, Faculty of Veterinary Medicine, Ankara,Turkey; [2]Department of Animal Medicine, Production and Health, University of Padova, Padova, Italy; [3]Livestock Central Research Institute, Ankara, Turkey.

Keywords: Angora goat, freezability, polyvinyl alcohol.

Small concentrations of the synthetic polymer polyvinyl alcohol (PVA) were found to inhibit the formation of ice in water/cryoprotectant solutions. Therefore, we aim to define a methodology to evaluate the freezability of Angora goat semen diluted with 3 different molecular weighted polyvinyl alcohol (PVA; 9, 18 and 100 kDa) with computer-assisted sperm analysis (CASA) before freezing in which each sperm head trajectory is reconstructed. In total, 30 ejaculates from seven mature Angora bucks (2 years old) were collected twice a week by artificial vagina. Immediately after collection, sperm samples were diluted with three different PVA co-polymers PVA 9, 18 and 100 kDa in with five different concentration 0,001 %, 0,01%, 0,1 %, 1% and %2 added to Tris-egg yolk diluent with 7% glycerol in three experimental groups respectively. After dilution semen was loaded into 0,25 ml French straws and cooled down to + 4C in three hours, frozen in a programmable freezing machine (Digitcool 5300, IMV, France). After thawing, following sperm motion characteristics were evaluated: Progressive motility and kinetic parameters with Hamilton-Thorn CASA, validated for buck semen analysis. Data collected and elaborated were analyzed using through ANOVA with PVA type and concentration as independent variable while CASA parameters as dependent variables. General post-thaw average of motility, progressivity, average pathway velocity (VAP), Straight line velocity (VSL), curvilinear velocity (VCL) and Lateral Amplitude were 55.76, 29.26, 121.84, 97.72 and 8.55 respectively. The mean differences (Δ) of motile percentages between the pre and post-thawed semen were 9.07, -9.73 and 14.58 and Δprogressive motility percentages were -24.71, -22 and -8.90 for three groups of PVA as 9 kDa, 18 kDa, and 100 kDa respectively. Lowest progressivity loss gained with group PVA 100 kDa along with ΔVAP, ΔVSL, ΔVCL and concordantly with ΔTotal rapid percentage. However, post-thaw motion trajectory/characteristics were better in PVA 18 kDa group average considering the lowest loss of ΔBeat cross frequency, ΔStraightness, and Δ Linearity. Δtotal static percentage was highest with PVA 9 kDa group ($P<0,05$). In conclusion, the addition of PVA 100 kDa was beneficial for sperm kinetic parameters. PVA 18 kDa group shown more proper motion characteristics. PVA addition to semen extender can decrease the glycerol concentration and thus can decrease the deleterious toxic effect. Synthetic PVA-derived ice blocking agents can be produced much less expensively than antifreeze proteins, offering new opportunities to improve the sperm cryopreservation.

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Ultrastructure of porcine embryos after cryopreservation

Z. Waligorski[1], B. Gajda*[2], Z. Smorag[2], M. Romek[1]

[1]Department of Cell Biology and Imaging, Institute of Zoology and Biomedical Research, Jagiellonian University, Krakow, Poland; [2]Department of Animal Reproduction Biotechnology, National Research Institute of Animal Production, Balice/Krakow, Poland.

Keywords: pig, embryo, cryopreservation.

In this study we examined the effect of cryopreservation on the ultrastructure of porcine embryos. It is considered that low survival of pig embryos after cryopreservation is related to a high content of lipid droplets (LDs) in their cytoplasm. In porcine zygotes these organelles occupy up to 60% of their volume (Romek et al., Reprod Domest Anim 44, 24-32, 2009). Cryopreservation can cause damages in the structure of LDs and mitochondria, and microsurgical LD removal enhances survival after cryopreservation (Kawakami M., Animal Reproduction Science 106, 402-411, 2008). Therefore, in the present study we focused on the morphology of mitochondria and LDs, which play a crucial role in embryo metabolism. Under a transmission electron microscope (TEM) we analyzed *in vivo* and *in vitro* derived embryos at three developmental stages: zygote, morula and blastocyst. Polish Large White gilts were artificially inseminated and embryos were collected surgically after flushing oviducts (zygotes) or uteri (morulae and late blastocysts). Additionally, part of the zygotes were cultured up to the morula and late blastocyst stage in the NCSU–23 medium supplemented with 4 mg/ml BSA at 39^{o} C in atmosphere containing 5% CO_2 in air. *In vivo* and *in vitro* derived control embryos were fixed immediately after acquisition with 2.5% glutaraldehyde in 0.67 M cacodylate buffer at 4^{o} C for 24 h, post-fixed in 1% osmium tetroxide, dehydrated in graded series of ethanol and embedded in PolyBed 812 epoxy resin (Polysciences Inc., Warrington, USA). The embryos were then cut into ultra-thin sections, contrasted with uranyl acetate and lead citrate and examined under the TEM. Experimental groups of embryos were vitrified using the Open Pulled Straw technique, thawed and then processed for TEM. All chemicals, unless otherwise stated, were from Sigma-Aldrich Co. (St. Louis, USA). In non-cryopreserved embryos differences in morphology of mitochondria between developmental stages were evident. In zygotes they were round shaped, contained a small number of cristae and a dark matrix. In morulae and blastocysts mitochondria were elongated, contained more cristae and a bright matrix. LDs were not disturbed. However, *in vivo* and *in vitro* embryos showed damages in ultrastructure after cryopreservation. Mitochondria contained bright vesicles and disturbed inner membranes, while in LDs we observed long cracks, often reaching the surface of organelles. Moreover, vitrification caused changes in LD surface, which was less homogeneous and contained bright areas with irregular edges. Mitochondrial functions including ATP production and calcium homeostasis can be disturbed due to the inner mitochondrial membranes disruption. Furthermore, LD cracks break the continuity of the lipid monolayer on the surface of LDs, which plays a crucial role in regulation of embryo lipid metabolism including lipolysis. Therefore, damages in embryo ultrastructure following cryopreservation may impair its developmental potential, leading to embryo death .

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