A226E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Comparisons of lipid content and genes of lipid metabolism in follicular cells and fluid in follicles of different size in bovine

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Keywords: Lipid metabolism, FA metabolism, follicle-size, mass spectrometry.

Ovaries of mammals have thousands of follicles intended for atresia and only a few become dominant and designated to ovulate. The energy cost for follicular growth is high and requires different substrates, including fatty acids (FA). Somatic follicular cells and oocyte have molecular machinery to metabolize FA into energy. Within bovine biotechnologies, oocytes of the large follicles are more competent for the in vitro embryo development compared to the small ones. The objective of our study was to elucidate the specificity of the lipid composition and the metabolism of FA in antral bovine follicles of different sizes. MALDI-TOF mass spectrometry (MS) imaging allowed the mapping of 281 lipid characteristics in ovarian compartments. Lipid analysis using Red Nile demonstrated differential size dependent distribution of neutral lipids in granulosa (GC) and theca (TH) cell layers. MALDI-TOF MS lipid fingerprints of isolated follicular cells and follicular fluid (FF) of small (SF, medium size 5 mm) and large follicles (LF, mean size 13 mm) acquired by MALDI-TOF MS revealed drastic changes in follicular fluid lipidome (more than 55% of the detected characteristics varied more than twice between LF and SF, Student's t-test, $P<0.05$). The size of the follicle significantly influenced the lipid composition of TH, GC and cumulus cells (ranged from 5%, 15%, and 10%, respectively) in contrast to oocytes that had less than 2% of lipid profile modulation between SF and LF. Identified differential lipids (in total 17%) revealed potential changes of membrane lipids in both somatic follicular cells and fluid along with follicular growth. Among them, the phospholipids containing long and very long chain FAs were preferentially found more abundant in the cells of the LF. In the oocytes from SF., two identified phosphatidylcholines (PC29:1, PC31:1) and 1 sphingomyelin (SM 32:1) were more abundant than in LF. Analysis of gene expression in TH and GC in LF compared to SF suggests a significant increase of FA beta-oxidation and oxidative stress, respectively (observed by expression of *ACADVL, HADHA,* and *GPX4*). Gene *ACOT9*, coding for a thioesterase catalyzing the hydrolysis of long-chain Acyl-CoAs, showed overexpression in TH of the SF. In summary, FA metabolism in follicular cells changes through follicular growth and significantly modulates lipid composition of FF. Differential distribution and abundance of lipids, including signaling molecules may, therefore, influence either follicular atresia or dominance; also, an increase in long-chain FA can provide substrates for post-ovulation body-luteal progesterone production.

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A new contribution to the improvement of human embryo culture media: a comparative study of low-abundance proteins of reproductive fluids and plasma of fertile women

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Keywords*:* low-abundance proteins, human reproductive fluids, salpingectomy.

The improvement of the embryo culture media is gaining relevance as demonstrated by the growing number of publications describing its influence on successful implantation rates, pregnancy, neonatal outcomes and potential effects in the adult life. The ideal conditions for embryo development are those naturally occurring in the female reproductive tract, i.e., the oviductal and uterine fluids. These fluids provide all the nutrients, hormonal and non-hormonal factors, electrolytes, macromolecules as well as precisely regulated volume, pH and osmolality required for the gametes, zygotes, and later, embryo development. In order to shed light on the differences between chemical and natural media, a detailed study of the composition of the female reproductive fluids is imperative. Here, we performed the first comparative study of the low abundance proteins in plasma, uterine and oviductal fluid collected from January 2016 until June 2018, simultaneously, from healthy and fertile women that underwent a salpingectomy. In order to select the most homogenous samples for this study, 3 women (out of 62 initially recruited) were selected based in the following criteria: similar age (31, 33 and 39 years old), evidence of healthy progeny and phase of their menstrual cycle (secretory phase). Samples were collected with a modified Mucat® device. The amount and quality of the collected samples allowed us to perform an efficient antibody-based depletion of the most-abundant serum proteins to facilitate the detection of the lower-abundance proteins of each fluid. The rationale for this design derives from the fact that high-abundant proteins in these fluids usually come from blood serum and usually mask the detection of low abundant proteins, which presumably could have a significant role in specific process related with the reproductive function. Differential regulation was measured using label-free quantitative shotgun proteomics, and statistical significance was measured using q-values (FDR). All analyses were conducted using software from Proteobotics (Madrid, Spain). The proteomic analysis by 1D-nano LC ESI-MSMS has shown a higher number of differentially expressed proteins in the oviductal fluid (131) than in the uterine fluid (22) when compared to plasma. From these 131 proteins, 92 were upregulated and 39 downregulated. Regarding the up-regulated proteins identified, they were predominantly involved in cellular catabolic processes, biosynthesis of aminoacids and organic substances, organic and aromatic compounds and catabolic acid signalling. The differentially expressed proteins of uterine fluid were mainly proteins implicated in immune response and granulocyte activation. In conclusion, this study presents a high-throughput analysis of female reproductive tract fluids, which constitutes a novel contribution to the knowledge of oviductal and uterine secretome.
Supported by MINECO-FEDER (AGL 2015-66341-R), 20040/GERM/16 and European Union, Horizon 2020 Marie Sklodowska-Curie Action, REPBIOTECH 675526.

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Transcriptome of porcine blastocysts stored in liquid state for up to 48 h

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Keywords: embryo, pig, liquid storage, transcriptome.

Recently, we have developed a method for the liquid storage of *in vivo*-derived porcine blastocysts. Storage is done at 25 ºC in NCSU-BSA medium without controlled CO_2 gassing for up to 48 h, thus facilitating the commercial application of embryo transfer (ET) in pigs. In a preliminary study with few ETs, 30% liquid stored blastocysts for 48 h were able to develop *in vivo* until day 38 of pregnancy. However, subsequent ET-studies with liquid-stored blastocysts, showed impaired farrowing rates compared to that of fresh or 24 h-stored blastocysts. Therefore, to elucidate the causes of these pregnancy losses, we evaluate hereby the transcriptional patterns of *in vivo*-derived blastocysts stored in liquid state for 24 and 48 h. Blastocysts were collected by laparotomy at Day 6 of the cycle (D0=onset of estrus) from weaned cross-bread sows (N=7). Some fresh blastocysts (control group) were frozen immediately after collection and stored at -80ºC until transcriptome analysis. The rest of the blastocysts were stored in 1 mL of NCSU-BSA in Eppendorf tubes at 25ºC for 24 or 48 h upon which they were morphologically evaluated under a stereomicroscope. A total of 30 viable blastocysts (three pools of 10 blastocysts) per group were transcriptomically analyzed. Transcripts (24,123) were evaluated in a microarray (GeneChip Porcine Genome Array, Thermo Fisher Scientific). A False Discovery Rate adjusted analysis p-adjusted <0.05 and a fold change cut-off of ±1 were set to identify differentially expressed genes. Data were analyzed using Partek Genomic Suite 7.0 software, which also identified altered KEGG pathways. None of the stored blastocysts had hatched by the end of storage. The blastocyst survival rates at 24 (97 %) and 48 h (94 %) of storage were similar to those achieved in control blastocysts (100%). However, the number of differentially expressed genes of stored blastocysts compared to controls dramatically increased during storage, from 127 genes by 24 h to 4,175 genes by 48 h). Blastocysts stored for 24 h displayed 70 down-regulated and 57 up-regulated genes. Only seven pathways (Axon guidance, PPAR signaling, Long-term potentiation) had an enrichment score >4, with less than 5% of their genes modified with respect to the control blastocysts. In contrast, in blastocysts stored for 48 h, 2,120 genes were down-expressed and 2,055 over-expressed. Thirty-six pathways had an enrichment score >4. In addition, 12 pathways showed more than 30% of their genes altered, related to pathways fundamental for embryonic development and pregnancy as: Protein processing in endoplasmic reticulum, Metabolic pathways, Cell cycle, Oxidative phosphorylation, Notch signaling pathways, Mismatch repair, Nucleotide repair and DNA replication. These results would not only certainly explain the very low pregnancy rates obtained with 48 h-stored blastocysts in our previous studies but also help designing novel target strategies to improve liquid storage systems for porcine embryos.
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A229E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Influence of different extenders on post-thaw quality of cryopreserved yak (Poephagus grunniens) semen

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Keywords: extender, post-thaw, semen, yak.

Yak is a unique livestock of high altitude surviving under hypoxic and extreme cold conditions above 3000 metre from mean sea level. They are considered as multipurpose animal as they provide milk, meat, fibre/wool, hide, fuel and the much needed transportation to the highlanders. Yak breeding face a lot of challenges under field conditions due to geographical isolation of the herds and repeated use of same breeding bull for generations. Cryopreservation of semen and use of Artificial Insemination (AI) can be one of the effective tools for overcoming the breeding problem in yaks. Yak semen has been successfully cryopreserved using Tris-citrate-fructose-egg yolk-glycerol (TFYG) extender (S. Deori, SAARC. J. Agric., 15, 215-218, 2017). Therefore, present study was designed with an objective to compare commercially available soybean based and liposome based extenders with TEYG extender on post-thaw quality of yak semen following cryopreservation. Semen was collected from 4 mature yak bulls (aged between 4 to 5 years) using artificial vagina. A total of twenty ejaculates (5/bull) having initial motility >70 percent were used for the study. Each ejaculate was splitted and diluted with BioXCell®, OPTIXCell® (IMV Technologies, France) and TFYG extenders to achieve a concentration of 30 x 10^6 per 0.5 mL straw. TFYG extender consists of 20 percent egg yolk and 6.4 percent glycerol. The straws were cooled to 5°C and equilibrated for 4 hours before freezing and cryopreserved in liquid nitrogen (BKD Borah et al., Int. J. Chem. Stud., 6, 509-511, 2018). The straws were thawed at 37°C for 10 seconds and evaluated for post-thaw sperm motility under phase contrast microscope (400x) and recorded from 0 to 100 based on the percentage of progressive motile sperm, sperm viability by Esoin-Nigrosine stain and acrosomal integrity by Giemsa stain. For sperm viability and acrosomal integrity a total of 200 sperm were counted and recorded in percentage. The mean values of the post-thaw sperm motility (%) in TFYG, BioXCell® and OPTIXCell® extenders were 53.50 ± 0.53, 54.50 ± 0.34 and 56.75 ± 0.55 respectively. The corresponding values for percent sperm viability and acrosomal integrity were 63.95 ± 0.53, 64.55 ± 0.51 and 66.35 ± 0.43, and 87.95 ± 0.67, 88.50 ± 0.66 and 91.10 ± 0.35 respectively. Analysis of variance indicated that post-thaw sperm motility, sperm viability and acrosomal integrity were significantly ($P<0.05$) higher in OPTIXCell® extender in comparison to BioXCell® and TFYG extenders. The values did not differ significantly between BioXcell and TEYG extenders. In conclusion, OPTIXCell® – a liposome based extender may serve as an alternative for successful cryopreservation of yak semen.

A230E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Combined embryo and recipient metabolomics improves pregnancy prediction in cattle

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Keywords*:* Embryo, recipient, metabolomics.

Pregnancy prediction within IVP embryos or recipients often fails, as competence of the embryo is not defined in recipient studies and vice-versa. We designed a multi-variate study with controlled factors (i.e. embryo breed, recipient breed, Day-6 embryonic stages) to identify pregnancy biomarkers in recipient plasma and embryo culture medium (CM; SOFaaci). Abattoir oocytes matured and fertilized with Asturiana de los Valles (AV) or Holstein sperm were first cultured in groups, and singly from Day-6 to Day-7 (12 µL CM). Expanded blastocysts were vitrified/warmed (V/W) and the CM was stored at -150°C until metabolomic analysis by GC-qTOF/MS. V/W embryos (N=24 [AV] and N=12 [Holstein]; N=6 bulls) were transferred to synchronized recipients (N=13 AV, N=17 Holstein and N=6 crossbred), and blood plasma was collected on Day-0 (PD0; N=35) and Day-7 (PD7; N=36). Independent Holstein embryos and recipients (N=13 ETs) were used for validation. Pregnancy was diagnosed on Day-62 and birth. Metabolites identified were N=36 (CM) and N=71 (plasma). Metabolite values in CM were subtracted from incubated blank controls. Data were transformed by Pareto scaling and weighed by embryo breed, bull and embryonic stage on Day-6 (embryos), and recipient breed. Pregnancy-regulated metabolite concentrations were identified by GLM (P<0.05 and FDR (P<0.05)). Biomarkers were obtained in two ways: 1) singly, by ROC-AUC>0.650 (P<0.05; FDR<0.05); and 2) by F1 score, as a Boolean product of 1 metabolite from CM and 1 metabolite from plasma. Thus, concentrations of metabolites in pregnant plasma and CM were paired (True (T)*T = T), and open samples outside the pregnant range considered T (one, another, or both, plasma and CM, outside the pregnant range) or false (F; both CM and plasma in the pregnant range). Biomarkers in CM at birth were capric acid (C, P=0.021) and monostearin (M; P=0.016); CM at Day-62: no metabolite. PD0 at birth: creatinine (CR, AUC: 0.690, P=0.024) and azelaic acid (AZ, AUC: 0.694, P=0.047). PD7 at Day-62: Leucine (L, AUC: 0.744, P=0.029). Fold changes in CM were >3.0, while in plasma were <2.0. Combining non-significant PD0 metabolites as glycine (G, 22 –correct- /35 –total- samples), hydrocinnamic acid (HY, 23/35) and hippuric acid (HI, 21/35) with the CM biomarker C improved sample classification (GxC: 28/35; HYxC: 30/35; HIxC: 28/35), and their respective F1 score (0.780, 0.872, 0.829; P<0.003) over C AUC (0.755; 23/36). Similar increases were observed with the CM biomarker M (not shown). In contrast, PD0 significant biomarkers as CR and AZ did not improve F1 accuracy as combined with C or M. Target metabolomics using pure analytical standards coinjection confirmed the identity of C, M, HY and HI in the analyzed samples. Validation of M in Holsteins led to AUC=0.769 (10/13), while C showed AUC=0.615 (8/13). Reliable birth predictions are feasible once: 1) biomarkers are identified in CM and plasma; and 2) embryo and recipient biomarkers are confronted. Acknowledgements: MINECO (AGL2016-78597-R and AGL2016-81890-REDT). GRUPIN 2018-2020 (IDI/2018/000178). FEDER. COST Action 16119 (Cellfit). ASEAVA. ASCOL.

A231E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Pregnancy after short exposure of cryopreserved porcine embryo to cryoprotective agents

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Keywords*:* porcine embryo, vitrification, short exposure.

The pig industry has nowadays an increasing demand for a reliable and cost-effective porcine embryo cryopreservation allowing long-term conservation, transport and widespread dispersion of high-quality genetics resources. Progress in embryo vitrification process made it possible to use the method in pigs, but lower and variable pregnancy rates are achieved with frozen embryos compared to fresh one. High concentrations of cryoprotective agents (CPAs) used for vitrification are believed to negatively affect developmental competence (Woelders et al, Cryobiology, 2018). The aim of the present study was to test the viability of cryopreserved porcine embryo after short exposure to CPAs during vitrification process. Embryos were surgically recovered 6 days after ovulation from Large White sows. Only embryos at the blastocyst stage were selected and vitrified in superfine open pulled straw (SOPS). Embryos were firstly placed in equilibration solution (ES) containing 7.5% ethylene glycol (EG) and 7.5% DMSO, and then in vitrification solution (VS), containing 16% EG, 16% DMSO and 0.4M sucrose. Embryos were incubated 2 min in ES and 30 sec in VS (short exposure to CPAs) or 3 min in ES and 1 min in VS (control, Cuello et al, RFD, 2010). Embryos were then loaded into straws and plunged into liquid nitrogen. After thawing, they were transferred to Talp-Hepes PVA with decreasing sucrose concentrations (0.13 and 0M) for 5 min each. In vitro and in vivo survival were tested. For in vitro survival, embryos were cultured for 3 days in 50µL of NCSU-23 + 10% FCS at 38.8°C in a humidified atmosphere of 5% CO_2 in air. For in vivo survival, embryos were surgically transferred in uterine horn of synchronised Meishan recipient (30 blastocysts per recipient). Three hundred and four embryos were used to test in vitro embryo survival after short and control exposure to CPAs. In the first experiment, the survival rate was better with shorter exposure to CPAs (66.2% vs 45.6%, p=0.008, n=145), but in the second experiment, it was identical (47.4% vs 60.6% ; p=0.101, n=159). New embryos were produced and collected (n=157) to test in vivo survival. Transfers were performed with embryos vitrified according to short exposure to CPAs. Among 4 recipients, one is pregnant. Farrowing is expected in next weeks. Our results show that a short exposure to CPAs is as efficient as the longer exposure usually employed for porcine embryo vitrification. As short exposure decreases the embryos contact with high level of toxic CPAs, reducing potential harmful and epigenetic modification of embryonic genome, this short exposure to CPAs should be chosen for porcine embryo vitrification.

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Cumulus-oocyte complexes-like 3D models to analyze sperm binding

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Keywords: zona pellucida, gamete interaction, magnetic beads.

The oocyte encapsulated by a glycoprotein matrix named zona pellucida (ZP) is surrounded by cumulus cells forming the cumulus oocyte complexes (COCs) that are ovulated in a plug in polytocous mammalian species. In recent years, a new 3D model to study gamete interaction in depth under *in vitro* conditions has been developed (Hamze, Animal Reprod 13: 647, 2016; Hamze, Animal Reprod 14 (3): 974, 2017). The model, consists of porcine ZP recombinant proteins conjugated to magnetic Sepharose® beads (B_{ZP}), supports sperm binding and resembles oocyte's size and shape being a valuable tool to simulate gamete interaction studies. In this work, we have taken a step forward to improve the model by better imitating the shape and possible function of the native COCs by incubating the B_{ZP} with cells cumulus thus generating cumulus-oocyte complexes-like 3D models ($CB_{ZP}C$) to evaluate whether they support further sperm binding. In order to obtain the $CB_{ZP}C$ models, B_{ZP} were generated as previously described with recombinant porcine ZP2, ZP3 and ZP4 proteins (Hamze, Animal Reprod 13: 647, 2016; Hamze, Animal Reprod 14 (3): 974, 2017). B_{ZP} were incubated for 24 h with cumulus cells isolated from *in vitro* matured porcine COCs (2,500 cells/ B_{ZP}). Then, groups of 50-55 $CB_{ZP}C$ were incubated for 2 h with fresh ejaculated porcine sperm separated by double centrifugation method (200,000 sperm/mL) at 38.5 ºC, 20% O_2, 5% CO_2, and saturated humidity. After co-incubation period, $CB_{ZP}C$ were washed twice in PBS, fixed and stained with Hoechst 33342. The mean number of sperm bound per $CB_{ZP}C$ ($S/CB_{ZP}C$) was scored by epifluorescence microscopy. Data was analyzed using Systat v13.1 (Systat Software, Inc San Jose, CA, USA) by one-way ANOVA and the values compared by Tukey's test when P value <0.05. The preliminary results (3 replicates) show that $S/CB_{ZP}C$ was significantly higher for the $CB_{ZP}C$ conjugated to ZP2 (11.88 ± 0.72, N=176) than beads conjugated to ZP3 (5.82 ± 0.41, N=195) and ZP4 (8.83 ± 0.61, N=176). These results are consistent with the ones previously reported with the 3D models without cumulus cells (B_{ZP}) (Hamze, Animal Reprod 13: 647, 2016) as well as the ones described in human and mice (Avella, J Cell Biol. 205(6):801-9, 2014; Avella, Sci Transl Med. 27;8(336):336ra60, 2016) suggesting that ZP2 could act as a sperm receptor in porcine species as well. In conclusion, this study offers a more physiological 3D model offering data consistent with previous observations thus reinforcing the viability of these models as a valuable tool to study gamete interaction. Moreover, as recombinant technology it is easily transferable to other species.

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Effect of highly dispersed silica nanoparticles on the functional activity of actin cytoskeleton in native and devitrified bovine oocytes during IVM

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Keywords*:* oocyte, cytoskeleton, vitrification.

Actin takes over various essential function during oocyte meiosis (UrajiJ, et al., J Cell Sci,131 22: 1-6, 2018). Nanoparticles are widely used in various fields including reproduction. The mechanisms of the influence of *highly dispersed silica nanoparticles* (HDSns) on the functioning of intracellular organelles are still not clear. The aim of the present study was to identify the effects of HDSns (Chuiko Institute of Surface Chemistry, Ukraine) on the functional activity of the actin cytoskeleton [the intensity of fluorescence of rhodamine-phalloidin (IFRF) conjugated with actin filaments] in dynamics of meiosis of native **(unfrozen)** and devitrified (DV) oocytes. IFRF was evaluated in: native oocytes; native oocytes were cultured with 0.001% of HDSns; DV oocytes; DV oocytes pre-treated with 0.001% of HDSns before vitrification (20 min) and were cultured with 0.001% of HDSns. Vitrification was performed by equilibration of cumulus oocyte complexes (COCs) **before IVM** in: CPA1:0.7 M dimethylsulphoxide (Me2SO) +0.9 M ethylene glycol (EG), 30 sec; CPA2:1.4 M Me2SO + 1.8 M EG, 30 sec; CPA3:2.8 M Me2SO + 3.6 M EG + 0.65 M trehalose, 20 sec and loading into straws. After thawing COCs washed in 0.25 M,0.19 M and 0.125 M trehalose in TCM-199 and finally in TCM-199. COCs were cultured 24 h in TCM 199 + 10% (v/v) FCS + 50 ng/ml PRL with 10*6granulosa cells /ml. For assessment of chromatin and IFRF fixed oocytes were incubated sequentially in rhodamine-phalloidin(RF, R415 Invitrogen, Moscow, Russia), 1 IU/ml, for 30 min to label actin. Then oocytes were incubated in 4',6-diamidino-2-phenylindole, 10 |µg/ml, for 10 min to label chromatin. Oocytes were examined using confocal laser scanning system Leica TCS SP5 with inverted fluorescent microscope. Diode 405 nm, argon 488 nm and helium-neon 543nm laser lines were used for fluorochrome excitation. IFRF were expressed in arbitrary units. All chemicals, except for RF, were purchased from Sigma-Aldrich (Moscow, Russia). Data were analyzed by ANOVA**.** Chromatin status and IFRF of 391 native and DV oocytes (in 3 replicates, 30-34 oocytes/group) were evaluated during IVM. There were no differences between the IFRF in native oocytes and native oocytes treated with HDSns before and in dynamic of culture (23±1.1 vs 21.1±1.08; 14 h of IVM - 55.8±5.6 vs 49.2±6,7; 24 h of IVM - 29.8±5,8 vs 21.3±7.3, respectively). The lowest level of IFRF were tested in DV oocytes before, after 14 h and 24 h of IVM (14.7±4.4, 16.1±3.8, 10.5±6.1, respectively). Treatment of DV oocyte with HDSns increased the IFRF after 14 h and 24 h of IVM (16.1±3.8 vs 37.8±5.9 and 10.5±6.1 vs 23.5±4.9, respectively, $P < 0.01$, $P < 0.05$). The data of study showed that the treatment of COCs with 0.001% of HDSn influences on actin cytoskeleton integrity of bovine oocytes during vitrification. The mechanisms of the realization of this effect are under the further investigation.
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A234E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and “omics”

Quality of bovine in vitro produced embryos derived from frozen-thawed oocytes

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Keywords*:* bovine, cryopreservation, in vitro fertilization.

Bovine oocyte cryopreservation has recently been a promising method of preserving genetic resources. A prerequisite for the proper development of preimplantation embryos after IVF is good quality of thawed oocytes. The aim of the study was to compare quality of IVP embryos derived from vitrified-warmed (V) oocytes with those obtained from fresh (C; control) oocytes. Embryo quality was evaluated basing on blastocyst cell number. Bovine cumulus-oocyte complexes were matured *in vitro* and then frozen by an ultra-rapid cooling vitrification technique in a minimum volume of vitrification solution (E-199 medium, 25 mM HEPES, 30 % ethylene glycol, 10% FBS) using 300 mesh electron microscopy nickel grids as a carrier. After warming the oocytes were fertilized in vitro and cultured in a Menezo B2 medium on the cumulus cell monolayer until the blastocyst stage. Embryo cleavage rate was counted on the Day 2 and blastocyst rate - on the Day 7 of embryo culture. For cell number counting, DAPI staining on nuclei observed under a Leica fluorescent microscope was used. For evaluation of differences between the experimental and control groups in embryo cleavage and blastocyst rate a Chi-square test, and in blastocyst cell number the t-test were used. Cleavage rate (Day 2) in V oocytes was lower (55.81%) compared to C (72.5%) group. Similarly, the blastocyst rate (D7) in V oocytes was different (11.82%) compared to C group (23%). In the V group, the higher incidence of asynchronous division at 3-cell stage, irregularly divided blastomeres at advanced embryo stages, and occurrence of blastocyst-like embryos (appearance of a blastocoel, but only a few nuclei) were observed in contrast to fresh oocytes. However, the cell number in truly D7 blastocysts in the V group (85 ± 10.16) was not statistically different from the control (97.3 ± 6.43) blastocysts. In conclusion, although the quality (the cell number) of V-derived blastocysts is comparable with those of fresh control, their development is affected by an asynchronous/irregular division resulting in a lower blastocyst rate. Further optimization of an oocyte cryopreservation regimen is required.
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Proteomic analysis of ejaculated and epididymal sperm associated with freezability in Iberian ibex (Capra pyrenaica)

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Keywords: proteome, small ruminants, spermatozoa.

The sperm proteome is known to affect cell cryoresistance and is reported to differ between epididymal and ejaculated sperm in small ruminants (C.J. Li *et al.*, Animal Reproduction Science, 173, 1–7, 2016; T. Pini *et al.*, Journal of Proteome Research, 15, 3700-11, 2016). However, studies aiming at identifying proteins involved on sperm freezing-tolerance are scarce. The aim of this study was to investigate the association between the freezing capacity and the proteome of ejaculated and epididymal sperm of the Iberian ibex. Ejaculates were collected from anesthetized animals by transrectal ultrasound-guided massage of the accessory sex glands combined with electroejaculation (n = 6), whereas epididymal samples were collected post-mortem by flushing (n = 6). After seminal/epididymal fluid removal, sperm cells were conventionally cryopreserved by slow freezing. Sperm quality parameters were assessed in fresh and frozen-thawed sperm to evaluate sperm freezability. Motility parameters were assessed by computer-assisted sperm analysis system and membrane and acrosome integrity were assessed by fluorescence microscopy. Tandem mass tag-labeled peptides were analyzed by high performance liquid chromatography coupled to a mass spectrometer (MS; Orbitrap Fusion Lumos) in three technical replicates. A false discovery rate of 1% was applied as protein identification threshold. The MS raw data were processed in Proteome Discoverer 2.2.0.388 and the statistical analysis was done using the moderated t-test of the R package limma. Epididymal sperm showed higher post-thaw total motility (57.46±8.58% *vs* 23.19±3.05%), progressive motility (37.70±6.38% *vs* 8.65±1.83%), curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) than ejaculated sperm ($P<0.0001$). Post-thaw acrosome (89.50±0.56% *vs* 61.95±3.48%; $P<0.001$) and membrane integrity (57.33±7.26% *vs* 35.73±3.39%; $P<0.05$) were also higher in epididymal sperm. A total of 1660 proteins were quantified in both epididymal and ejaculated samples among which 310 proteins (18.7% of the total) were differentially expressed between both types of sperm when using a cut-off for significance (adjusted p-value <0.05) and fold-change (abs(log2 (fold-change)>1). Out of those proteins, 212 were significantly more abundant in epididymal sperm and 98 were more abundant in ejaculated sperm. Peroxiredoxin-4 (PRDX4) and superoxide dismutase [Cu-Zn] (SOD1) are proteins involved in cell protection against oxidative stress and were more abundant in epididymal than ejaculated sperm. Heat shock protein HSP 90-alpha (HSP90AA1) is a chaperone involved in structural maintenance and cell cycle control that was also more abundant in epididymal sperm. Besides updating the sperm proteome of small ruminants, this study revealed differences of cryoresistance between epididymal and ejaculated sperm of the Iberian ibex contributing to identification of candidate markers of sperm freezability.

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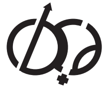

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Effect of different egg yolk extenders on in vitro fertility of thawed ram semen

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Keywords: thawed semen, egg yolk extenders, in vitro penetration.

An attempt to optimize sperm freezing extenders in ram was made by the replacement of fresh egg yolk (FEY) by powdered egg yolk (PEY). Therefore, ejaculates from 8 rams (2 years old) were collected by artificial vagina and mixed immediately. Pooled semen was centrifuged twice and the pellet was split into two aliquots and resuspended in a Tris-citric acid-glucose solution with 5% glycerol and 15% of PEY or FEY for 4 h at 5ºC before freezing in liquid nitrogen vapours. Secondly, cumulus-oocyte complexes were obtained by slicing from prepubertal sheep ovaries, selected and matured in 100 µL drops of BO-IVM® medium (IVF Bioscience, UK) plus estrous sheep serum (ESS, 10%) for 24 h at 38.5ºC in 5% CO_2 atmosphere. Then, FEY and PEY semen samples were thawed and spermatozoa were selected by 2 mL density gradient (40%/80%), using BoviPure® and BoviDilute® (Nidacon, Mölndal, Sweden) for its preparation. Once the column was centrifuged at 1200g for 10 min, the supernatant was discarded, the pellet resuspended with 2 mL of BoviWash® and then centrifuged again at 1200g for 5 min. After discarding the supernatant, total and progressive motility was assessed from the selected sperm populations by a CASA system (ISAS® , PROISER SL, Valencia, Spain). Then, the selected sperm were co-cultured with the in vitro matured oocytes in 100 µL drops of BO-IVF® medium (IVF Bioscience, UK), supplemented with 2% ESS, at a final concentration of $2x10^6$ sperm/mL. After 17 h of co-culture, oocytes inseminated (n=126 for FEY and n=127 for PEY) were mechanical decumulated, washed and fixed in 4% paraformaldehyde (v/v) at room temperature for 1 h approximately. Afterwards, oocytes were stained with Hoechst 33342 for 15 min in the dark at 4ºC. Then, oocytes were mounted on slides and kept at 4ºC in darkness until analysis under an epifluorescence microscopy (ZEISS Axioskop 40, Oberkochen, Germany). The parameters assessed were the penetration rate (number of oocytes penetrated by at least one spermatozoon of the total number of the potential mature oocytes) and the monospermy rate (number of oocytes penetrated by a single spermatozoon of the total penetrated oocytes). Statistical analysis was performed using a General Lineal Model procedure (SPSS® 20, IBM® Corporation, Armonk, NY, USA). The results (mean ± SD) did not show significant differences between extenders on penetration rate (82.6 ± 6.7 and 88.2 ± 3.9, for FEY and PEY, respectively) neither on monospermy rate (58.5±13.6 and 60.4±13.4, respectively). As well, no significant differences were found between extenders on total sperm motility (41.6±13.1 and 37.1±12.1 for FEY and PEY, respectively) and progressive motility (14.9±5.3 and 9.2±5.6 for FEY and PEY, respectively). In conclusion, powdered egg yolk can substitute successfully the conventional fresh egg yolk in ram sperm freezing extenders, providing higher biosecurity, due to its pasteurization process, and greater homogeneity in the composition of the diluents.

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Incubation with seminal plasma after thawing reduces immature sperm in Blanca de Rasquera goats

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Keywords*:* cryopreserved sperm, seminal plasma, male age.

Addition of seminal plasma (SP) to cryopreserved sperm has been suggested as a potential alternative for improving sperm quality after thawing. DNA status, measured by the Sperm Chromatin Structure Assay (SCSA) provides an accurate measurement of sperm with a high DNA stainability (HDS), which has been related to sperm immaturity. Based on this, a preliminary study was conducted to evaluate the addition of SP to post thawed goat sperm on HDS levels in cryopreserved sperm from 1 and 2 years old bucks, thus studying a potential male age effect. Briefly, ejaculates from 8 bucks were collected via artificial vagina twice a week in 2 consecutive breeding periods. Fresh ejaculates were immediately pooled, centrifuged twice and diluted in a Tris-based media containing 15% powdered egg yolk (NIVE, Nunspeet Holland Eiproducten, Ochten, The Netherlands). Then, diluted samples were refrigerated for 4 h at 5°C before freezing in liquid nitrogen vapours. After thawing at 37°C for 30 seconds, sperm samples were selected by a single layer centrifugation using BoviPure® (Nidacon, Mölndal, Sweden), according to the manufacture instructions. Then, selected sperm sample was split in 2 aliquots and incubated in different media consisting on: a) *in vitro* fertilization commercial media (BO-IVF, IVF Bioscience, UK) and b) BO-IVF media plus 20% SP, for 3 h at 38,5ºC in a 5% CO_2 atmosphere at a final concentration of $40x10^6$ sperm/mL. Then, HDS sperm proportion was determined using flow cytometry after acid-detergent treatment for 30 s and acridine orange staining according to SCSA methodology described by Evenson et al (Journal of Andrology, 2002; 23, 1, 25-43). Viability was also assessed by flow cytometry using SYBR14 and PI probes. Statistical analysis was performed using a General Lineal Model procedure (SPSS 19.0). Results showed no significant differences on HDS sperm proportion (mean % ± SE, n=6) between samples collected from 1 (4.4±0.8) and 2 years old males (3.7±0.6) after incubation for 3h in BO-IVF media. Likewise, no differences were found between sperm samples from 1 (3.0±0.6) and 2 years old males (1.6±0.2) after 3h incubation in BO-IVF media plus seminal plasma. However, this HDS population was significantly lower in sperm from 2 years old males incubated in the presence of seminal plasma compared to the sperm incubated only in BO-IVF media, while in 1 years old males no significant differences were observed. However, neither the male age nor the incubation media had a significant effect on thawed sperm viability, showing values (mean % ± SE) of 12.0±2.6 and 16.6±4.8 in 1 year old male samples and 15.3±3.6 and 14.8±1.8 in 2 years old male sperm incubated in BO-IVF or BO-IVF+SP for 3h, respectively. In conclusion, this study showed that the age of the donors had no identifiable beneficial effect on the HDS-parameters in goat sperm. Further studies are needed to elucidate the potential beneficial effect of seminal plasma on HDS levels in cryopreserved, especially in older male sperm. Supported by INIA (RZP2014-00001-00-00) and PIVEV (AGL2016-81890-REDT).

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Intracytoplasmic sperm injection using sex-sorted sperm in the bovine

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Keywords: ICSI, paternal contribution, cattle.

Intracytoplasmic sperm injection (ICSI) involves the fertilization of oocytes at the metaphase II stage (MII) by direct injection of a sperm cell. Despite the similar success rates between ICSI and conventional IVF in humans, the success rates in cattle are poor with low developmental rates compared to those obtained via IVF. One reason might be the need of an artificial activation of the oocyte after sperm injection. This activation could also lead to parthenogenetic embryonic development up to the blastocyst stage and beyond without paternal contribution. Checking the morphology of the obtained blastocysts is not sufficient to distinguish between parthenogenetic and fertilized embryos after ICSI. Therefore, we used Y-sorted sperm for ICSI. Sexing the subsequent blastocysts employing bovine and Y-chromosome specific primers will provide reliable results regarding the sex of the embryos. Bovine cumulus-oocyte-complexes collected from follicles out of slaughtherhouse ovaries were in vitro matured employing a standard protocol (Stinshoff et al. Reprod Fertil Dev. 2014;26(4):502-10). After denudation, only MII oocytes were used for ICSI. Bovine Y-sorted sperm from four (bull 1-4) different bulls (1.5-2 years of age) with proven fertility (ICSI Y1, Y2, Y3, Y4) was prepared via a SpermFilter™ centrifugation. After immobilization of the sperm cell, it was sucked into the ICSI pipette using Eppendorf manipulators mounted on an Olympus microscope and injected into the oocyte. Chemical activation was performed with 5 µM ionomycin for 5 minutes followed by a 3 h culture period and an additional incubation for 3 h in 1.9 mM 6-DMAP. After activation, oocytes were cultured in SOFaa for 8 days. Oocytes which have been fertilized conventionally by co-culturing oocytes and sperm (IVF), which have only been activated (CA) and those which have been injected with non-sorted (ICSI non) and X-sorted (ICSI X) sperm served as controls. Experiments were repeated at least four times with an average of 25 oocytes per run. Cleavage (day 3) and developmental rates at day 8 (mean±SD) ranged from 15.8±5.4 to 40.9±13.1 % and 0.0 to 11.0±3.6 % in the ICSI Y1-Y4 groups. Within the control groups of embryos the following data could be obtained: 66.7±9.7, 33.0±7.4 % (IVF); 44.0±11.9, 9.7±3.5 % (CA); 49.2±18.0, 11.6±10.6 % (ICSI non); 51.4± 7.7, 7.1±2.8 % (ICSI X). After sexing, only 4 of the tested 21 embryos stemming from the ICSI Y1-Y4 group were male. Only 1 male embryo could be obtained using non- sorted semen for ICSI (1/24 embryos tested). Embryos out of the CA (16/16) and ICSI X groups (3/3) were all female as expected. After IVF, 38 out of the total number of 59 tested embryos were female. Taken together, these data indicate that there is a bull-specific suitability of sperm to be used for ICSI. Furthermore, even when an advanced developmental stage has been reached, the paternal contribution needs to be verified.
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Histological cut of a paraffin-embedded blastocyst: optimized protocol for murine blastocysts

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Keywords: blastocysts, Histological-cuts, paraffin-embedded.

Paraffin-embedded tissues have been used for research and therapeutic applications for decades, as they represent a valuable tool in histology and for molecular analysis, as well as being a way to preserve tissue samples for long periods at a low cost. The information currently available to embed blastocysts into paraffin blocks include: protocols using gelatin and paraffin, protocols to embed a piece of uterus containing the blastocyst already implanted, and a few protocols for *Xenopus* or bovine embryos, using specific equipment that might not be available in every laboratory. Nonetheless, little information is available on easy protocols to embed mouse blastocysts into a paraffin block to then make histological cuts. The purpose of this work was to create an optimized protocol to embed mouse blastocysts into paraffin blocks, without using gelatin, and to perform histological cuts of the sample with the morphology well preserved, which can then be used for subsequent analysis. For this protocol we used 3 in vivo mouse blastocysts and performed 20 cuts in total with the microtome. Each one of them was fixed with 4% PFA for 30 min, permeabilized with 0.2% of Triton X-100 for 30 min, stained with 2 % eosin for 15 seconds to facilitate its visualization, dehydrated using different concentrations of ethanol (96% and absolute) for 10 min each, immersed in xylene for 5 min and embedded into the paraffin block. The next day, the paraffin block was cooled down and cut into 6 µm sections with a microtome, and the sample was processed to remove the paraffin, stain the cellular structures and be visualized under a microscope. Out of the 20 histological cuts performed, 65% of the sections contained just some cells of the blastocyst or the blastocyst folded onto itself, but the protocol was optimized based on the results and problems encountered until good histological cuts of the blastocyst with the morphology well preserved were obtained. Sections were considered of good quality when the samples had a circular shape and the nucleus and cytoplasm of the cells could be identified. This optimized protocol can be used to obtain good quality histological sections of a blastocyst, which can be used for studies involving *in situ* hybridization, immunohistochemistry, enzyme histochemistry, DNA, RNA or protein extractions, analysis of biomarkers, characterization of surface markers of stem cells integrated into the embryo, to analyze the effect of potential compounds that could be used to improve the embryo culture media, to prepare histological material for educational purposes, etc. Some of these studies could represent a valuable source of new information for the field of reproductive biology.