Animal Reproduction

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

SUPPORT BIOTECHNOLOGIES: CRYOPRESERVATION AND CRYOBIOLOGY, DIAGNOSIS THROUGH IMAGING, MOLECULAR BIOLOGY, AND "OMICS"

Thymus satureioides essential oil and antibiotics: Evaluation of their effect on the quality of frozen thawed Beni Arouss bucks semen

Amr Kchikich[1,6], Nathalie Kirschvink[2], Sara El Kadili[3], Marianne Raes[4], Samira El otmani[1], Youssef Chebli[1], Jean- Loup Bister[4], Bouchra El amiri[5], Said Barrijal[6], Mouad Chentouf[1]

[1]National Institute of Agricultural Research, Regional Center of Agricultural Research of Tangier, Rabat, Morocco
[2]Department of Medicine, Namur Research Institute for Life Sciences (NARILIS), University of Namur, Namur, Belgium
[3]Department of Animal Production, Ecole Nationale d'Agriculture de Meknès, Meknes, Morocco
[4]Department of Veterinary Medicine, Namur Research Institute for Life Sciences (NARILIS), University of Namur, Namur, Belgium
[5]National Institute of Agricultural Research, Regional Center of Agricultural Research of Settat, Rabat, Morocco
[6]Laboratory of Biotechnological Valorization of Microorganisms, Genomics, and Bioinformatics, Faculty of Sciences and Techniques, University Abdelmalek Essaadi, Tangier, Morocco
e-mail: kchikich.amr@gmail.com

Keywords: Beni Arous buck semen, cryopreservation, Thymus satureioides

The objective of this study was to examine the effect of different concentrations of *Thymus satureioides* (TS) essential oil (EO) and antibiotics on the quality of post-thawed Beni Arouss buck semen. TS EO is known for its antioxidant and antibacterial properties. When incorporated at 0.01% to skim milk–based extender, TS EO proves beneficial in enhancing the preservation of buck semen at 4°C. Semen collection was conducted using an artificial vagina once a week for ten weeks from eight Beni Arouss bucks. The ejaculates were combined, divided into six equal aliquots, and washed before being diluted to 400 x 10^6 sperm/ml with 7% glycerol. Skim milk-based extender was supplemented with different concentrations of TS EO (0%, 0.01%, and 0.05%) without antibiotics, denoted as (CTR-), (S1-), and (S5-), and with 50 mg of streptomycin and 50,000 IU of penicillin per 100 ml, marked as (CTR+), (S1+), and (S5+), respectively to assess the improvement of sperm preservation efficiency by combining them with TS EO. The aliquots were cooled to 4°C, then frozen in 0.25 ml straws using a programmable freezer and ultimately stored in liquid nitrogen. Thawing was performed at 37°C for 30 seconds. Post-thawed semen quality parameters, including motility, live sperm, membrane integrity, lipid peroxidation, and bacterial growth, were evaluated. Sperm motility was assessed using the computer-assisted sperm analysis system, viability was determined through eosin-nigrosin staining, membrane integrity was evaluated using the hypo-osmotic swelling test, lipid peroxidation was measured by the thiobarbituric acid reactive substance assay, and bacterial growth was quantified by calculating the number of colony-forming units per milliliter in a non-selective culture medium. The obtained data was checked for normality using the Shapiro-Wilk test, and all data were not normally distributed. The non-parametric Kruskal-Wallis test was used to assess the impact of antibiotic or concentration of EO, and the means were compared using the Steel-Dwass-Critchlow-Fligner test in case of a significant effect. A probability value of ($p < 0.05$) was considered statistically significant for all parameters. These statistical analyses were carried out using SAS 9.4. S1+ treatment resulted in a lower bacterial growth and lipid peroxidation compared to CTR+ ($P < 0.05$), but motility, viability, and integrity parameters remained the same ($P > 0.05$). On the other hand, when assessing the impact of antibiotics, it was observed that the CTR+ and S1+ improved all quality and bacterial growth parameters compared to CTR- and S1- respectively, but resulted in a significant increase in malondialdehyde formation. However, 0.05% of TS EO showed a toxic effect, regardless of the presence or absence of antibiotics. Based on the findings, the use S1+ extenders are recommended to enhance the cryopreservation of Beni Arouss buck semen.

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In vitro production of bovine embryos modified sex-dimorphisms in fetal liver methylome.

Véronique Duranthon[1], Aurélie Chaulot-Talmon[1], Francesca Ali[1], Ludivine Laffont[1], Anne Frambourg[1], Derya Sebukhan[1], Olivier Desnoës[2], Eugénie Canon[1], Aurélie Bonnet[3], Valentin Costes[3], Marjolaine André[1], Beatrice Mandon-Pépin[1], Pascal Salvetti[2], Laurent Schibler[3], Hélène Jammes[1]

[1]Université Paris-Saclay, UVSQ, INRAE, BREED, Jouy en Josas France
[2]Station de phenotypage, Eliance, Nouzilly, France
[3]Eliance, Jouy en Josas, France
e-mail: veronique.duranthon@inra.fr

Keywords: IVP/IVD fetuses, methylome, sex-effect

Environmental perturbations of early mammalian embryo affect adult phenotype in a sex-specific manner. This assumes that sex-specific differences exist even before hormonal impregnation, due to differences in sex chromosomes between males (M) and females (F). Because long term effects of embryo environment are thought to be mediated by alterations of the epigenome, we asked to what extent the epigenome of a fetal organ differs between M and F and whether these differences are altered by *in vitro* production of the embryos. Therefore, we compared liver methylomes of M and F bovine fetuses developed from *in vivo* derived (IVD) and *in vitro* produced (IVP) embryos at Day 40, that is before any hormonal impregnation. Thirteen IVD fetuses were recovered from 22 synchronized females at Day 40 post insemination. IVP fetuses developed from embryos produced from slaughterhouse ovaries. Embryos were cultured in SOF medium supplemented with 1% oestrus cow serum, then transferred fresh at Day7 to 38 synchronized recipient females, fetuses were recovered 33 days later to produce 14 Day 40 IVP fetuses. All IVD and IVP fetuses were recovered from euthanized females and fetal livers were dissected. Genomic DNA was extracted, fetuses were sexed by PCR. A total of 21 fetuses (5 individuals of each sex and development condition, but 6 IVD males) were kept for further analyses. Liver DNA was submitted to Reduced Representative Bisulfite Sequencing (RRBS) to profile genome-wide methylation on a single nucleotide level. RRBS analysis was performed using RRBS-toolkit pipeline (Perrier et al. 2018 BMC Genomics) including Methylkit software to identify differentially methylated cytosines (DMCs). Sites for known Single Nucleotide Polymorphisms were filtrated before differential analysis. Only cytosines with a 10-500 coverage were selected for analyses. A threshold of 25% difference in methylation rate and an adjusted p-value<0.01 were retained to select DMCs. We compared DMCs between male and female fetuses developed either from IVD or from IVP embryos. A total of 1543 DMCs were found between IVD M and IVD F fetal livers, most of which (n=1324) on the X chromosome which was not unexpected after X inactivation. In vitro production increased by a factor of 1.7 the total number of DMCs (n=2734) both on X (n=2407) and on autosomes (n=327). While most (91%) of the X-linked DMCs were hypermethylated, those located on autosomes were equally distributed between hyper and hypomethylation in IVD female livers. Interestingly, this was not the case in IVP female livers where 97% of the X-linked and 76% of the autosomal DMCs were hypermethylated. These results point to an hypermethylation of the most sex-dimorphic DMCs in IVP female fetal livers. DMCs were similarly distributed between genes and repeated sequences in IVP and IVD livers but they were slightly enriched in CpG islands in IVP compared to IVD livers (82.5% and 74.7%, respectively). Work is going on analysing methylomes of fetal gonads, chorion, and brain, and integrating transcriptome and methylome data.

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Astaxanthin during post-warm recovery period improved quality of bovine blastocysts obtained from vitrified oocytes

Linda Dujíčková[1,2], Lucia Olexiková[1], Alexander V. Makarevich[1], Alexandra Bartková[2,3], Lucie Němcová[3]

[1]Research Institute for Animal Production, National Agricultural and Food Centre (NPPC), Nitra-Lužianky, Slovak Republic
[2]Faculty of Natural Sciences and Informatics, Constantine the Philosopher University in Nitra, Nitra, Slovak Republic
[3]Institute of Animal Physiology and Genetics of the Czech Academy of Sciences, Liběchov, Czech Republic
e-mail: linda.dujickova@nppc.sk

Keywords: astaxanthin, oocytes, vitrification

Exogenous antioxidants in maturation and culture media are widely used to mitigate the negative effect of vitrification on oocytes. Effect of astaxanthin (Ax) on bovine oocyte cryosurvival has not been documented. The aim of this study was to evaluate the impact of Ax added during post-warm recovery on the developmental competence of bovine vitrified oocytes. Oocytes aspirated from slaughtered-derived cow ovaries were matured *in vitro* (IVM; M199, 10% FBS, 0.25 mmol·L^{-1} sodium pyruvate, 50 µg/mL gentamicin, 1 I.U FSH/LH (Pluset)) at 38.5 °C and 5% CO_2, partially denuded and vitrified in M199 medium with 30% ethylene glycol, 1 M sucrose and 10% FBS in minimum volume on the electron microscopy grids by ultra-rapid vitrification technique. Vitrified/warmed oocytes were incubated 3 hours for post-warm recovery in the IVM medium either with (V-Ax-2.5 µM; n = 226) or without (V-Ax-0; n = 258) Ax. Fresh IVM oocytes (n = 201) served as a control. Afterwards, oocytes were fertilized *in vitro* using frozen bull semen. Presumptive zygotes were incubated in a B2 Menezo medium with 10% FBS, 10 mg/mL BSA, 50 µg/mL gentamicin and 31.25 mM sodium bicarbonate on a monolayer of BRL-1 (Rat epithelial cells; ECACC, UK) cells at 38.5 °C and 5% CO_2 until the blastocyst stage (6-8 days). Experiments were performed in 5 replicates. Relative fluorescence intensity of post-warmed oocytes showed significant (ANOVA; $p < 0.05$) increase of ROS level (CellROX™) in V-Ax-0 group (3.69 AU) compared to control (1 AU), while Ax significantly decreased ROS level (2.08 AU) in vitrified oocytes. Similar trend was observed in relative fluorescence intensity of lipid peroxidation (BODIPY™). Ax reduced lipid peroxidation in vitrified oocytes (0.66 AU), which was caused by oxidative stress due to vitrification (1.38 AU). Significant decrease (Chi-squared test; $p < 0.05$) was observed in cleavage and blastocyst rates in both V-Ax-0 (58.14% and 14.34%) and V-Ax-2.5 (54.87% and 17.26%) groups compared to control (66.17% and 29.85%). However, blastocysts from V-Ax-2.5 group had significantly (t-test; $p < 0.05$) higher total cell number (103.80 ± 2.81) compared to V-Ax-0 group (94.03 ± 5.08) and was comparable to control (105.28 ± 4.45). RT-qPCR assay showed significantly (t-test; $p < 0.05$) higher expression of the *GJB5* gene in Ax group compared to control, confirming the proliferative effect of Ax. Significantly lower expression of *CAT* and *GPX4* genes was detected in the V-Ax-0 group, while Ax increased expression of these genes compared to control. Expression of the apoptotic *CAS9* gene was increased due to vitrification, while Ax suppressed it to the control level. *CDX2*, *SOD2*, *BAX* and *BCL2* gene expression was not affected by either vitrification or astaxanthin. In conclusion, astaxanthin during post-warm recovery period reduced oxidative stress in bovine vitrified oocytes and improved quality of blastocysts up to those from fresh oocytes.

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Decellularization of female domestic cat's reproductive organs: ovary, oviduct and uterine horn

Phakjira Sanguansook[1], Cristina Martínez-López[2,3,4], Mª José Izquierdo-Rico[2,3], Carlos Martinez-Cáceres[3], Kaywalee Chatdarong[1], Francisco Alberto García-Vazquez[3,4]

[1]Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand
[2]Departamento de Biología Celular e Histología, Facultad de Medicina, Universidad de Murcia, Murcia, Spain
[3]Instituto Murciano de Investigación Biosanitaria Pascual Parrilla (IMIB), Murcia, Spain
[4]Departamento de Fisiología, Facultad de Veterinaria, Universidad de Murcia, Murcia, Spain
e-mail: phakjira.san@gmail.com

Keywords: decellularization, cat, 3D scaffold

Decellularization is an innovative method to create natural scaffolds by removing all cellular materials but maintaining the composition and three-dimensional (3D) ultrastructure of the extracellular matrix (ECM). Obtention of decellularized reproductive organs in cats might facilitate the development of assisted reproductive technologies in feline species. The objective of our research was to compare the efficiency of three decellularization protocols (P1, P2 and P3) in reproductive organs (ovary, oviduct, and uterine horn) in domestic cats. P1 consists of two cycles of decellularization (Campo et al., Biology of Reproduction, 96(1), 34–45, 2017). P2 was similar to P1, but samples were decellularized by means of three cycles. P3 was similar to P2 but the samples were additionally incubated with deoxyribonuclease for 24h at 37 °C. Reproductive organs from nine cats were separated into two sides. One side was the control (non-decellularized organ) while the contralateral was the treatment (decellularized organ). The treatment group was subdivided into 3 groups (n=3 per each group) for entering P1, P2 or P3. Both control and treatment samples were individually analyzed for DNA content, histology (for evaluating collagen, elastin and glycosaminoglycans (GAGs)), and scanning electron microscopy (SEM). DNA content from different protocols were compared using Kruskal-Wallis test with pairwise comparison. Histology results were compared using unpair t-test or Mann Whitney test. Statistical significance was considered when $P<0.05$. The successful removal of nuclear material was confirmed by DNA quantification. The results of the study showed that DNA concentration between control and treated samples after P1 and P2 had no significant difference. However, there was a significant difference between control and treated sample after P3 of each organ. Regarding to ECM content level in control ovaries and decellularized ovaries from each protocol were not significant different. On the other hand, decellularized oviducts demonstrated a significant decreased level of collagen and GAGs in P3 when compared with the control group. Elastin also significantly decreased in decellularized oviduct from P2 compared with control. Decellularized uterine horns demonstrated a significant decreased level of collagen and GAGs in P3, however elastin level remained constant in both controls and decellularized uterine horns for each protocol. SEM revealed that the microarchitecture of the decellularized samples were maintained when compared with controls. In conclusion, when comparing different decellularization methods, P3 was more efficient than P1 and P2 for removal of nuclear material in reproductive organs. P3 demonstrated its successfulness to decellularize ovarian samples by significantly decreased DNA content, maintained ECM contents and maintained tissue microarchitecture. However, P3 was less effective to maintain ECM contents of decellularized oviducts and uterine horns. This method can be further developed for creating 3D ovarian scaffold for follicle culture in this species.

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Molecular alterations in oviductal epithelial cells during heat stress in dairy cows

Konstantina Magkrioti[1], Konstantina Stamperna[2], Themistoklis Giannoulis[1,3], Eleni Dovolou[2,3], Zissis Mamuris[1], Katerina Moutou[1], Georgios Amiridis[2]

[1]Laboratory of Genetics, Comparative and Evolutionary Biology, Department of Biochemistry and Biotechnology, University of Thessaly, Greece
[2]Department of Obstetrics and Reproduction, Veterinary Faculty, University of Thessaly, Karditsa, Greece
[3]Department of Animal Science, University of Thessaly, Larissa, Greece
e-mail: konamagrioti@gmail.com

Keywords: oviductal epithelial cells, gene expression, dairy cows

Climate change, and especially the rise of temperature leads to heat stress, which is a major threat for dairy cows' fertility due to induced alterations of the endocrine status, increased oxidative stress phenomena, reduced fertilizing capacity of the oocyte and increased embryo deaths. The oviduct and its secretions (the oviductal fluid) play a crucial role in fertility and early embryo development. In this study, the spatiotemporal effect on gene expression during a thermoneutral and a heat stress period was examined. The estrous cycles of ten Holstein cows were synchronized during a thermoneutral and a HS period. On day 3 of the cycle, the cows were slaughtered in groups of 3 or four, the oviducts were immediately removed and transferred in the lab within two hours. In each season, the epithelial cells were collected by scraping the oviductal epithelium with a glass slide and the content collected from the ipsilateral and contralateral to the corpus luteum oviducts was pooled. From 4 replicates in each season, total RNA was extracted and reversed transcribed and Real Time PCR was performed in genes that are involved in response to oxidative and heat stress, under stress regulation of transcription, ER stress response, development, growth and apoptosis. The results were analyzed using a two-way ANOVA with a post-hot Tukey test and a Pearson correlation was calculated for each pair of genes, to detect coordinated patterns of gene expression in each season. Anova test showed that season affected significantly the expression of *EIF2A* in the ipsilateral oviduct and *SOD2* expression was altered independently of location. Both genes exhibited higher expression during the summer. *SOD2* contributes to the antioxidant mechanism of the cells while *EIF2A* is a major target of the unfolded protein response (UPR) and acts as a translation regulator. *HSP90AA1A* and *HSF1* genes' were up - regulated during the summer, though the differences were not statistically significant (p=0.08). Concerning the coordinated patterns of gene expression, a group of genes (*ATF6*, *ERN1*, *PPP1R15B*, *EIF2A*) was detected, having noticeably stronger positive correlation in the summer than in the spring. These genes participate in signaling pathways which suppress protein translation under conditions of stress. In conclusion, we provide evidence that HS induces antioxidant mechanism which protect the cells from ROS and maintain cellular integrity. Also, HS response and UPR are tightly regulated during summer. We infer that these regulations facilitate the proper folding of misfolded proteins, which along with the attenuated translation precludes further accumulation of misfolded proteins.

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Efficiency of immersion-agitation decellularization method on pig uterus: histological analysis of fresh and frozen-thawed tissue

Cristina Martínez López[1,2], Carlos Manuel Martínez Cáceres[3], María José Izquierdo Rico[2,3], Francisco Alberto García Vázquez[1,3]

[1]Department of Physiology, Veterinary School, University of Murcia, Spain
[2]Department of Cell Biology and Histology, Faculty of Medicine, University of Murcia, Spain
[3]Institute for Biomedical Research of Murcia (IMIB-Pascual Parrilla), Murcia, Spain
e-mail: cristina.martinez67@um.es

Keywords: Decellularization, uterus, histology

Decellularization is a technique used to remove cellular and nuclear material from tissues and organs, resulting in a decellularized extracellular matrix (ECMd) that can serve as a biological scaffold for various applications. These include the regeneration of damaged tissues through recellularization with stem cells (Deepak Choudhury. Acta Biomater. 115:51-59, 2020), improvement of culture *in vitro* embryos (Hannes Campo. Acta Biomater. 89:126-138, 2019), and the creation of hydrogels for three-dimensional printing of tissues and organs (Abaci A. Adv Healthc Mater. 9(24): e2000734, 2020). This study aimed to evaluate the efficiency of the decellularization technique in uterine sections from porcine species, comparing fresh and frozen-thawed samples subjected to different decellularization cycles (0-0h cycle vs. 1-24h cycle vs. cycle 2-48h) by the immersion-agitation method. Histological analysis was performed on the samples to assess the success of the decellularization process. The decellularization procedure was carried out on 5.28 ± 2.82 cm long uterine (horn) sections from prepubertal porcine female (Large white). A total of 12 uteri were used (n=4 for each treatment: control (non-decellularizated tissue), fresh or frozen-thawed). Samples taken at the end of each decellularization cycle (Hannes Campo. Biol Reprod. 96(1):34-45, 2017) were fixed in formalin (4%, 24h). Tissues were embedded in paraffin and histological sections 5 µm-thick were stained with hematoxylin-eosin, Masson's trichrome, orcein, and alcian blue for viable nuclei, collagen, elastic fibers, and glycosaminoglycan (GAG) analysis, respectively. Nucleus counting was carried out on a total of 10 fields at 400x, and for collagen fibers, elastic fibers and GAG determinations, five fields at 200x were analyzed. Representative images were obtained with a Pannoramic MIDI-II automatic slide digitizer (Budapest, Hungary) and for image analysis a freeware image analysis software (ImageJ, NHI, USA) was used. For statistical analysis, the Kruskal-Wallis test or ANOVA One-Way was performed. The histological results demonstrated a significant reduction in the number of nuclei between cycle 0 (620.90 ± 33.11 for the fresh treatment and 551.40 ± 30.65 for the frozen treatment) and cycle 2 in both cases, fresh and frozen organs (8.30 ± 8.30, $p<0.01$ and 3.80 ± 2.17, $p<0.01$, respectively). For the rest of the parameters evaluated only significant differences were observed in collagen content between cycle 0 (53.90 ± 5.01%) - cycle 1 (27.45 ± 7.59%, $p=0.03$) and between cycle 0 - cycle 2 (23.97% ± 12.03, $p=0.02$) of the uteri subjected to fresh treatment. There were no significant differences in the percentage of elastic fibers and GAGs between cycle 0 and cycle 2, with $p>0.05$ in all cases. In conclusion, the decellularization protocol used in this study was effective in both fresh and frozen-thawed porcine uteri. However, frozen-thaw treatment preserved the extracellular matrix components better than fresh treatment.

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Effect of Cryotop simultaneous vitrification of large batches of pig blastocysts on miRNAs expression

Cristina Cuello[1,2], Alejandro Gonzalez-Plaza[1,2], Manuela Garcia-Canovas[1,2], Inmaculada Parrilla[1,2], Maria A Gil[1,2], Emilio A Martinez[1,2]

[1]Department of Medicine & Animal Surgery, Faculty of Veterinary Medicine, International Excellence Campus for Higher Education & Research (CMN), University of Murcia, Murcia, Spain
[2]Institute for Biomedical Research of Murcia (IMIB-Arrixaca), Murcia, Spain
e-mail: ccuello@um.es

Keywords: pig, blastocyst, vitrification, miRNA

MicroRNAs (miRNAs) are non-coding RNAs of 20-25 nucleotides that regulate post-transcriptional gene expression. The miRNAs are modulated by genetic and environmental factors and play important roles in embryonic development and implantation. Little is known about the effects of vitrification on embryo miRNA expression, and no information is available in pigs. The aim of this study was, first, to evaluate the effects of simultaneous vitrification of 20 porcine blastocysts using the open Cryotop device on the miRNA profile and, second, to link the results to previous gene expression data (Gonzalez-Plaza et al., in press, Theriogenology, 2023). Embryos were surgically collected from 12 donor sows on day 6 of gestation. A total of 120 blastocysts were selected for this experiment. Blastocysts (n=60) were vitrified in groups of 20 using the open Cryotop procedure as described before (Gonzalez-Plaza et al., Front Vet Sci, 9:936753, 2022). Control were non-vitrified blastocysts (n=60). Blastocysts from each donor were equally distributed between both groups. Postwarmed and control blastocysts were cultured in vitro for 24 hours to assess viability. The miRNA profile of some viable embryos (n=40 from each group; 5 different pools of 8 blastocysts each) was studied with the GeneChip miRNA 4.1 array of Affymetrix (ThermoFisher scientific, Madrid, Spain). Transcriptome Console 4.0 and Partek Genomic Suite software were used to analyze array data, the interaction with gene expression and biological interpretation. A threshold of 1.5 foldchange and $p<0.05$ were used to identify differentially expressed (DE) miRNA and genes (DEGs). Analysis of miRNA transcriptome was made using reference mammalian species (*Bos taurus, Equus caballus, Homo sapiens, Monodelphis domestica, Macaca mulatta Macaca nemestrina, Mus musculus, Ornithorhynchus anatinus, Pongo pygmaeus, Pan troglodytes, Rattus norvegicus and Sus scrofa)*. The viability of postwarmed (98.3%) and control (100%) blastocysts was similar at the end of the culture. In vitrified blastocysts, a total of 174 miRNAs exhibited differential expression compared to controls. Among them, ssc-miR-7139-3p from sus scrofa was upregulated, while the remaining 173 miRNAs, including ssc-miR-214 and ssc-miR-885-3p from sus scrofa, were downregulated. Under our knowledge, none of these three-miRNA annotated for sus scrofa have been previously described in pig embryos. The integrative analysis showed that 61 of the previously investigated DEGs in Cryotop-vitrified blastocysts compared to controls were regulated by some of these DE miRNAs. These target genes (TGs) significantly enriched the following KEGG pathways: HIF-1 signaling pathway (ARNT, GADPH, MKNK2; all upregulated TGs), Notch signaling pathway (HES1 and LFNG; both downregulated TGs), Ascorbate and aldarate metabolism (MIOX; upregulated TG), and Glycosphingolipid biosynthesis-ganglio series (SLC33A1; upregulated TG). Among them, Notch signaling pathway has been demonstrated as essential for embryo and fetal development. The biological impact of these results after embryo transfer requires further research.

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Proteome characterization of endometrium during implantation in the domestic pigs

Maria A Gil[1], Josep M Cambra[2], Heriberto Rodriguez-Martinez[3], Cristina Cuello[1], Inmaculada Parrilla[1], Emilio A Martinez[1]

[1]Dpt. Medicine and Animal Surgery, International Excellence Campus for Higher Education and Research, University of Murcia, Murcia, Spain
[2]Technical University of Munich. Munich, Germany; [3]Department of Biomedical & Clinical Sciences (BKV), BKH/Obstetrics & Gynaecology, Linköping University, Linköping, Sweden
e-mail: mariagil@um.es

Keywords: pig, proteome, endometrium

Characterization of the porcine endometrial proteome is important for understanding the complex embryo-maternal communication required for successful pregnancy outcome. It is during the implantation stage when most porcine embryos from natural or artificial breeding undergo death. Therefore, the aim of the present study was to characterize the proteomic profile of the "pregnant" endometrium at day 18 and 24 post insemination compared to that of the non-pregnant endometrium at the same days of the cycle. For this purpose, sows were inseminated postcervically with 1.5 x 10^9 live spermatozoa (for pregnant sows; PS) or dead spermatozoa (for non-pregnant sows; NPS). Sows were slaughtered to obtain endometrial tissue samples from three different attachment sites on day 18 of gestation (n=4 sows) or day 24 of gestation (n=4 sows). In NPS sows, endometrial samples were randomly collected from three different areas of the uterine horn on day 18 (n=4 sows) or day 24 of the cycle (N=4 sows). All samples were subjected to proteomic analysis to generate a data-dependent spectral library by LC-MS/MS. This analysis allowed the identification of 3254 and 3457 proteins in the endometrium of PS and NPS, respectively; of these, 1753 were common and 1501 and 1704 were unique to PS and NPS, respectively. Analysis of PS-unique proteins by functional GO analysis using the UniProtKB database and Cytoscape ClueGO™ Pathway Enrichment Analysis, revealed an interaction of CCR5, HMOX1, IFI35, ISG15, LBP, MAP2K1, MAPK14, SLA, STXBP2, VAMP7 proteins involved in immune system process in the network of cytokine signaling in immune system and CD14, DNM1, LBP, MAP2K1, MAPK14, S100A9 proteins in the network of Toll Like Receptor 4 cascade pathways. The analysis also revealed the interaction of EMILIN1, HAPLN1, ITGAM, TNC proteins involved in biological adhesion annotation in the extracellular matrix organization pathway. Regarding the identified PS-unique proteins involved in reproductive process annotation, ClueGO showed the interaction of DLG1, MAP2K1, MAPK14 proteins in T cell receptor signaling KEGG pathway and CCT3, CCT7 proteins in Folding of actin by CCT/TriC pathway. ClueGO analysis also revealed the involvement of PS-unique proteins with reproductive process annotation in various GO terms of Biological Process such as placenta development (HECTD1, HSD17B2, MAP2K1, MAPK14, RP56) or reproductive structure and system development (HECTD1, HSD17B2, MAP2K1, MAPK14, RP56, MSH2, DLG1, INHBB). This study provides relevant immune- and binding proteins that may play an important role in endometrial development during weeks 3 to 4 of pregnancy. The identification of these proteins may help to clarify the crosstalk between the endometrium and the conceptus at that critical period of pregnancy, when major conceptus loss occurs.

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Animal Reproduction

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

SUPPORT BIOTECHNOLOGIES: CRYOPRESERVATION AND CRYOBIOLOGY, DIAGNOSIS THROUGH IMAGING, MOLECULAR BIOLOGY, AND "OMICS"

In silico-designed vitrification protocols using propylene glycol for bovine oocytes as an alternative to dimethyl sulfoxide based protocols

Judith Diaz-Muñoz[1], Sonia Gago[1], Adam Z Higgins[2], Tania García-Martínez[1], Iris Martínez-Rodero[1], Teresa Mogas[1]

[1]Autonomous University of Barcelona, Spain
[2]School of Chemical, Biological and Environmental Engineering, Oregon State University, USA
e-mail: judit.diaz@uab.cat

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The cryopreservation of bovine oocytes is a widely employed technique in reproductive biotechnologies. However, it still faces significant limitations. Although several strategies have been implemented to optimize and refine vitrification protocols, there still remain shortcomings. The most extended protocols for bovine oocyte vitrification are based on a combination of ethylene glycol (EG) and dimethyl sulfoxide (Me_2SO), which have been demonstrated to provide acceptable outcomes in terms of post-warming survival, spindle configuration, and embryo development. Nevertheless, Me_2SO is associated with high cytotoxicity that can negatively impact oocyte competence. To address this concern, propylene glycol (PG) has been suggested as an alternative cryoprotectant that can be combined with EG to enhance vitrification outcomes. Therefore, the objective of study was to determine the optimal exposure time of IVM bovine oocytes to the equilibration solution containing EG and PG at two different temperatures (25°C and 38.5°C). The permeability values to EG and PG were assessed by using *in vitro* osmotic observations and mathematical modeling. For this purpose, IVM bovine oocytes (10 to 16 per group) were exposed to a concentration of 1,55 M PG or 1,55 M EG in TCM199-Hepes + 20% FBS at either 25°C or 38,5°C and incubated for 10 min (3 biological replicates). The volumetric response of the oocyte was recorded every 5 s with a time-lapse video recorder. The permeability parameters hydraulic conductivity (L_p) and CPA permeability (P_s) of the oocyte cell membrane were determined by fitting the experimental data to a two-parameter transport formalism. Then, *in silico*-predictions were obtained from the permeability data as previously described (García-Martínez et al. Theriogenology, 184, 110-123, 2022). *In silico*-predictions accuracy was tested by *in vitro* assessing the time required for the IVM oocytes to reach the osmotic equilibrium volume when exposed to ES composed by 7,5% PG + 7,5% EG in TCM199 medium + 20% FBS at 25 or 38,5ºC. Data were statistically analyzed by Shapiro–Wilk for normality and Levene's test for homogeneity of variance followed by one-way ANOVA and Tukey test ($p<0,05$). Results (mean±SEM) showed that the L_p (mm/atm*min) was significantly higher for PG than EG at both temperatures: 25ºC: EG 1,4±0,1, PG 2,7±0,2 and 38,5ºC: EG 2,3±0,2, PG 5,3±0,3. Also, PG showed higher ($p<0,05$) P_s (mm/s) at 38,5ºc than for EG (2,7±0,2 and 1,9±0,2, respectively) while no differences between CPAs were observed at 25ºC (PG:0,7±0,02; EG:0,8±0,1). In general, both permeability parameters were higher at 38,5°C than at 25°C at both CPAs. *In silico* predictions showed that original oocyte cell volume recovery is reached within 1 min 25 sec at 38,5°C and at 3 min 55 sec at 25°C. Results suggest that PG combined with EG could be used as a replacement of Me_2SO in bovine oocyte vitrification protocols due to its permeability characteristics and lower toxicity.

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