A276E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

The use of different progestin devices in ovarian stimulation protocol affects gene expression in sheep cumulus-oocyte complexes (COC)

G.M. Bragança[1], R.I.T.P. Batista[1], C.V. Souza[1], J.D.R. Santos[1], E.K.N. Arashiro[1], J.F. Fonseca[2], F.Z. Brandão[1], J.M.G. Souza-Fabjan[1]

[1]Universidade Federal Fluminense, Nitéroi, Rio de Janeiro, Brazil, [2]Empresa Brasileira de Pesquisa Agropecuária - Unidade Caprinos e Ovinos, Sobral, Ceará, Brasil.

Keywords: oocyte, progestogen.

Progesterone (P_4) and its analogues (progestins) are commonly used in estrus synchronization protocols and to hold the LH surge during ovarian stimulation to allow oocyte recovery in live donor ewes. However, recent evidence suggests that some progestins may have a deleterious effect on embryo quality after long-term use. Thus, the present study aimed to evaluate the effect of two progestin devices used during ovarian stimulation on the COC quality in donor ewes. A total of 30 pluriparous ewes had their estrus and follicular wave synchronized by a short-term protocol (Bragança et al., Reprod., Fertil. Dev., published online, 2018). At 80 h after sponge removal, all ewes received 80 mg of pFSH (Folltropin-V, Bioniche Animal Health, Ontario, Canada) in three applications (50%, 30% and 20%) every 12 h. For stimulation, the ewes were allocated into three groups (n = 10 each): MAP, in which ewes received intravaginal sponges containing 60 mg of medroxiprogesterone acetate (Progespon, Zoetis, São Paulo, Brazil); P_4, in which a silicone device impregnated with 0.33 mg of natural P_4 (CIDR, Eazi-Breed, Zoetis) was applied; and Control, in which the ewes did not receive any device (only luteal P_4). COCs were recovered by laparoscopy and morphologically graded as viable (GI/II, homogeneous ooplasm and at least a complete cumulus cells layer; and GIII, homogeneous ooplasm and/or partially denuded) or poor quality (GIV, heterogeneous ooplasm or degenerated). To infer development competence, viable COCs were stained with brilliant cresyl blue (BCB) and classified as BCB^+ (competent) and BCB^- (non-competent). Pools of 10 BCB^+ COCs/group were used for gene expression analysis by real-time PCR of oocyte competence markers (ZAR1, zygote arrest 1; MATER, maternal antigen that embryo requires; GDF9, growth differentiation factor 9; BMP15, bone morphogenetic protein 15; RELN, reelin; Bcl-2, B-cell lymphoma 2; and BAX, Bcl-2 associated X protein) and steroidogenic pathway-related genes (ERα, estrogen receptor α; LHr, LH receptor; FSHr, FSH receptor; and StAR, steroidogenic acute regulatory protein). An ANOVA was then conducted to compare the variables followed by a Tukey test. No significant difference ($P > 0.05$) was observed for the number of viable COCs per ewe (MAP: 5.7 ± 1.0, P_4: 7.7 ± 0.7 and Control: 5.7 ± 1.1) or the percentage of BCB^+ (MAP: 61%, P_4: 58% and Control: 65%). However, the gene expression profile was affected by the type of progestin used. FSHr, LHr and RELN genes were up-regulated ($P < 0.05$) in the P_4 as compared with the MAP group, while LHr and RELN genes were down-regulated in the MAP as compared with the Control ($P < 0.05$). Finally, FSHr, LHr, Erα, as well as the Bcl-2, ZAR1 and GDF9 were up-regulated in the P_4 as compared with the Control group ($P < 0.05$). In conclusion, the progestin device alters the expression of genes related to quality and the steroidogenesis pathway in fully-grown COCs, and the use of a natural P_4 device may improve the development competence of COCs. Further studies including IVP are necessary to confirm our findings.

A277E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

Analysis of correlation between zona pellucida birefringence using polarized light microscopy and resistance to trypsin digestion in different mammals

P. Cots[1], J. Sánchez-Férez[2], E. Gómez[3], L. Gónzalez-Brusi[1], M.J. Izquierdo-Rico[1], R.T. López-Donaire[1], J. Ballesta[1], M. Avilés[1]

[1]Department of Cell Biology and Histology, University of Murcia, IMIB-Arrixaca CMN, Murcia, Spain; [2]IMFER, Murcia, Spain; [3]SERIDA, Gijón, Spain.

Keywords: Zona pellucida, Hardening, Birefringence.

The Zona Pellucida (ZP) is an extracellular matrix of mammalian oocyte and early embryo. It plays physiologically important roles in fertilization including binding of spermatozoa and prevention of polyspermic penetration. Sensitiveness of ZP to protease digestion and resistance to enzymatic solubility is species-specific. Polarized light microscopy allows monitoring the multilaminar ZP structure and identifying three differentiated ZP layers. This study investigated whether retardance and thickness of the inner, middle and outer layers of different species determines resistance to trypsin digestion. Immature oocytes from bitch (n = 6), cow (n = 10), mouse (n = 5), pig (n = 16) and rat (n = 9) were collected from ovaries and analysed by polarized light microscope (polscope). The retardance and width of each layer of the ZP was analysed and measured by Oosight Meta® software (CRI, Woburn, MA, USA). Oocytes were incubated with trypsin solution (5mg/mL diluted in PBS) at 37ºC (Merck KGaA, Darmstadt, Germany). The time of ZP digestion was recorded and correlated with the birefringence and thickness outcomes. Analyses between groups were performed through a general lineal model, followed by Bonferroni's post-hoc test ($P < 0.05$). Pearson's correlation between species and parameters was analysed. The ZP of dog, mouse and rat showed lower ZP resistance to trypsin digestion (35.32 ± 0.80 min, 71.38 ± 2.16 min, 38.00 ± 1.49 min, respectively) compared to cow (111.09 ± 3.90 min) and pig ZPs (403.14 ± 23.84 min). As hypothesized, the inner, middle and outer layer were observed in bitch, cow, mouse, pig and rat oocytes. In all species the inner layer showed the highest birefringence. Furthermore, birefringence and thickness values differed between different species. Similar to ZP resistance, the relative thickness of inner ZP layer of pig (56.7%) and cow (56.8%) were higher than inner ZP layer relative width of bitch (47.6%), mouse (30.3%) and rat (47.7%). Finally, pig, cow and bitch showed higher birefringence, measured as retardance value, (5.98 ± 0.24nm, 8.83 ± 0.21nm and 6.75 ± 0.30nm, respectively) than mouse (0.97 ± 0.07nm) and rat (1.24 ± 0.10nm) ZPs. No significant correlations between ZP resistance to trypsin digestion and ZP birefringence or thickness were found in any analysed species. In conclusion, differences were observed among the ZP of different species related with the ZP protein organization using the polscope microscopy. No correlations were observed between trypsin digestion and polscope analysis.
However, an increased number of oocytes should be analysed to confirm these preliminary results.
Supported by MINECO AGL2015-70159-P and FEDER.

A278E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

Comparison of survival rates of two thawing methods for vitrified biopsied bovine *in vitro*-produced blastocysts

N. González[1], J. Scherzer[1], M. Reichenbach[1], H. Zerbe[2]

[1]Bayern-Genetik GmbH, Landshut, Germany; [2]Ludwig-Maximilian University, Munich, Germany.

Keywords: biopsied embryos, vitrification, survival rate.

When embryos are genotyped, pregnancy rates after transfer are lower due to the need for biopsy and cryopreservation. The main objective of this study was to assess *in vitro* survival rates of biopsied vitrified *in vitro*-produced blastocysts using two different vitrification devices and a one- vs. three-step warming system. Embryos were produced by a standard *in vitro* protocol and placed in the culture medium (synthetic oviductal fluid (SOF) supplemented with 5% oestrus cow serum (OCS), 40µl/ml basal medium Eagle (BME) and 10 µl/ml non-essential amino acid solution (MEM), at 38.5ºC in 5% CO_2 and 5% O_2). A total number of 215 of grade 1 blastocysts (IETS codes 6 and 7) were randomly divided into a biopsy group (BG, n = 76) and a control group (CG, n = 139). Blastocysts were biopsied using a microblade mounted on a micromanipulator. A small portion of the trophoblast, approximately 15%, was cut off. Both groups were then vitrified using the Cryotop ® (Kitazato, Tokio, Japan) or the new VitTrans device (Morató and Mogas, Cryobiology, volume 68, issue 2, pages 288–293, 2014). For vitrification, all blastocysts were exposed to an equilibration medium with 7.5% EG + 7.5% DMSO in holding medium (HM), consisting of TCM-199 with 20% fetal calf serum, moved into a drop with 16.5% EG + 16.5% DMSO + 0.5M sucrose in HM and then placed in a microdroplet on the Cryotop ® (Biopsy, n = 39; Control, n = 94) or VitTrans device (Biopsy, n = 37; Control, n = 45). Warming of embryos using the VitTrans device for vitrification occurred in a one-step procedure by incubating them at 38.5ºC in 0.5M sucrose solution for 5 minutes. Embryos vitrified with the Cryotop system were warmed using the conventional three-step procedure by incubating them at 38.5ºC in 1M sucrose solution for 1 min, 0.5M sucrose solution for 3 min and 0M sucrose (HM) for 5 min. After warming, all embryos were placed in the culture medium. Morphology and re-expansion were evaluated 24h post-warming. The embryo survival rate was defined as the ratio of blastocysts that were able to re-expand with regards to the total number of warmed blastocysts. A 67% survival rate was observed in all biopsied embryos compared to controls (71%) (SAS, PROC GLM; p-value = 0.5319). The ratio of re-expanded embryos of the 3-step warming system was 73% (biopsy group (69%) and control group (75%), Cryotop, n = 133), in comparison with 63% using the 1-step warming system (biopsy (65%) and control group (62%), VitTrans, n = 82); (SAS, PROC GLM; p-value = 0.1123). In conclusion, the biopsied embryos showed good *in vitro* development after vitrification and warming. However, based on our results, additional research is required to increase the re-expansion rate of embryos using the one-step warming procedure. The viability of the embryos needs to be confirmed by successful establishment of pregnancies.
This work was sponsored by Bayern-Genetik GmbH. We are thankful for the support of Dr. Huber and his team at Südfleisch Waldkraiburg GmbH, as well as Teresa Mogas at UAB.

A279E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

Design of pJuno-beads to study the molecular mechanisms of sperm-oocyte interaction

J.G. Hamze[1], R. Romar[2], M. Jiménez-Movilla[1]

[1]Departamento de Biología Celular e Histología, Facultad de Medicina, Universidad de Murcia, IMIB, Murcia, España; [2]Departamento de Fisiología, Facultad de Veterinaria, Murcia, España.

Keywords: Porcine Juno, conjugated beads, gamete interaction.

Juno, an essential protein in mammalian fertilization, was identified as the receptor for Izumo1 on mouse eggs and proven that the interaction between both proteins is conserved within mammals (Bianchi, Nature 508(7497): 483–487, 2014). Moreover, we have developed an *in vitro* 3D model mimicking oocyte's properties to study the molecular mechanisms involved in gamete interaction in pigs (Hamze, Animal Reprod 13: 647, 2016; Hamze, Animal Reprod 14 (3): 974, 2017). Thus, the objectives of this study were i) to construct expression plasmids encoding the sequence of porcine Juno (F1STK4), ii) to express this recombinant protein in mammalian cells and, iii) to create a new 3D model consisting of magnetic beads (His Mag Sepharose™ Excel) conjugated with porcine pJuno. cDNA encoding nucleotide sequence (672 bp) of Juno protein in *Sus scrofa* was kindly donated by Dr. Enrica Bianchi (Cell Surface Signalling Laboratory, Wellcome Sanger Institute, UK). pJuno was PCR-amplified with reverse primer contained a sequence encoding a 6 histidine-tag and cloned into a pcDNA3.1 (+). pJuno-Cherry (pcDNA3.1 (+)) was designed including mCherry fluorescent protein in-frame with 3' region of pJuno and tagged with 6-histidines before stop codon (GeneArt, Life Technology). Expression plasmids were verified by DNA sequencing and purified. Both proteins were *in vitro* expressed in Chinese Hamster Ovary cells (CHO) and, once secreted, identified by SDS-PAGE and western blot. Juno-Cherry expression was monitored by fluorescence microscopy. Then, the secreted recombinant proteins were conjugated with the magnetic beads. Groups of 60-65 beads conjugated to Juno were co-incubated for 1 and 2h with a heterospermic dose of boar spermatozoa in TALP medium at a final concentration of 200.000 spermatozoa/mL. After co-incubation, the beads were washed twice in PBS, fixed and stained with Hoechst 33342. The percentage of beads with at least one sperm bound (BSB) and the mean number of sperm bound per bead (S/B) were scored by fluorescence microscopy. Secreted proteins and their adhesion to the beads was confirmed by western blot with anti-His antibody. Recombinant pJuno showed a molecular weight of ≈30kDa and pJuno-Cherry ≈50kDa. The BSB rate was similar at both incubation times, being 96.1 ± 1.7% at 1h (n = 128) and 92.8 ± 2.5% at 2h (n = 111). The S/B was 15.0 ± 1.2 and 16.0 ± 1.4, respectively after 1 and 2h co-incubation. In conclusion, in this study, recombinant pJuno and pJuno-Cherry were *in vitro* expressed and identified for the first time. Moreover, the 6 histidine-tag of the recombinant protein allowed its successful conjugation to the magnetic beads generating a new and promising 3D model. Our preliminary results show that boar spermatozoa binds to Juno-beads but further studies are necessary to characterize this sperm-egg interaction and to gain more knowledge on the molecular basis of this interaction in pigs.

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".

A280E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

Effect of vitrification on the functional activity of mitochondria in porcine oocytes during *in vitro* maturation

T. Kuzmina[1], T. Stanislavovich[1], D. Tatarskaya[2]

[1]Russian Research Institute of Farm Animal Genetics and Breeding - Branch of the L.K. Ernst Federal Science Center for Animal Husbandry, St.Petersburg-Pushkin, Russia; [2]EmbryLife Center of Reproductive Technologies, St.Petersburg, Russia.

Keywords: oocyte, mitochondria, vitrification.

Problems in development an effective technology for vitrification of female gametes are determined by the features of oocytes structure, the sensitivity of cellular compartments to the action of ultralow temperatures, the toxicity of cryoprotectants. The aim of the present study was to evaluate the effect of pre-treatment of cumulus-oocyte complexes (COCs) with the fluid from the follicles (FF) d ≤3mm before vitrification on the mitochondrial activity (MA) in native and devitrified oocytes. MA was evaluated in: native oocytes; native oocytes pre-treated with FF (120 min, 37°C); devitrified oocytes; devitrified oocytes pre-treated with FF. Vitrification was performed by equilibration of oocytes 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 in maturation medium (NCSU 23) supplemented with 10% FF (d≤3 - 6 mm), 0.1 mg/ml cysteine, 10 IU/ml eCG and 10 IU/ml hCG at 38.5°C in a humidified atmosphere containing 5% CO_2. COCs cultured in maturation medium with pieces of follicle's wall (600-900 μm in length, Abeydeera L, et al., Biol Reprod. 58:213-218,1998). After 22 h of culture COCs and pieces of wall were washed and transferred into the same maturation medium but without hormonal supplements for next 22 h of culture. MA was measured by fluorescence probe MitoTracker Orange CMTM Ros, intensity of fluorescence expressed in μA/oocyte, chromatin status was evaluated with Hoechst 33342 (H.Torner et al., Reprod Dom Anim 42, 176–183, 2007). Oocytes were examined using confocal laser scanning system Leica TCS SP5. Chemicals were purchased from Sigma-Aldrich (Moscow, Russia). Data were analyzed by Student's t-test. Chromatin status and MA of 467 oocytes were evaluated (in 4 replicates, 27-30 oocytes/group). Before cultivation level of MA in native oocytes was significantly higher than in devitrified oocytes (331 ± 18,9 μA and 321 ± 17,7 μA vs. 105 ± 10, 3 μA and 150 ± 18,9 μA, $P < 0.01$). Pre-treatment oocytes with FF increased level of MA in diplotene, metaphase-I and II devitrified oocytes (105 ± 10,3 μA vs. 150 ± 18,9 μA, 149 ± 12,1 μA vs. 209 ± 9,9 μA and 117 ± 17,1μA vs. 155 ± 11,9 μA, $P < 0.05$). There were no differences in the level of MA at metaphase II stage of native and devitrified oocytes that have pre-treated with FF (158 ± 12,8 μA vs. 155 ± 11,9 μA). Features of the functional activity of mitochondria in native and devitrified oocytes during IVM have been identified. Treatment of porcine oocyte with FF (d≤3 mm) prior to vitrification had a positive effect on the mitochondrial function in devitrified oocytes. The obtained results supplement data concerning the functioning of mitochondria in porcine oocytes at the influence of ultralow temperatures.

This study was supported by the FASO, Russia (Project #181180215901329) and RFBR (Project #18-016-00147A).

A281E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

Cryodamage of oocytes frozen in antral follicles of bovine ovarian tissue fragments

A.V. Makarevich, L. Olexikova, M. Foldesiova, E. Kubovicova, J. Pivko

Research Institute for Animal Production Nitra, National Agricultural and Food Centre, Luzianky-near-Nitra, Slovakia.

Keywords: cryopreservation, oocytes, antral follicles.

Cryopreservation of ovarian tissues is largely developing method. Successful preservation of primordial follicles was documented in many animal species. There are reports that also oocytes in the antral follicles of ovarian fragments can be successfully cryopreserved, despite the fact that the cavity with follicular fluid is a barrier for freezing. The aim of our study was to assess histological and ultrastructural status of oocytes from small antral follicles cryopreserved in the ovarian fragments in order to judge about their developmental competence. Ovarian fragments (n = 451; approximate size of 4x4 mm), containing antral follicles (2-4 mm), were isolated from undefined cows at a local abattoir, and frozen by two methods previously used for freezing of ovarian primordial follicles. For solid surface vitrification (SSV), ovarian fragments were exposed to 4% ethylene glycol (EG) in DPBS+10% FBS for 15 min and then rinsed in a vitrification solution composed of 35% EG and 0.4 M trehalose in DPBS+10% FBS. After 5 min equilibration in an ice bath, fragments were placed in a minimum volume of vitrification solution onto the surface of a metal plate pre-cooled by an immersion into a liquid nitrogen (LN). For liquid vitrification (LV), ovarian fragments were equilibrated in a vitrification medium containing 40% EG, 30% Ficoll 70, 1M sucrose and 4 mg/ml of BSA at room temperature for 5 min. Then the tissues in 1.8 ml cryovials were placed into LN. After thawing the fragments were processed for histology and ultrastructure analyses; part of oocytes were tested for the ability to mature *in vitro* (IVM). After 24 h of IVM in maturation medium (TCM 199 (Gibco), sodium pyruvate (0.25 $mmol.l^{-1}$), gentamycine (0.05 $mg.ml^{-1}$), fetal bovine serum 10% and FSH/LH (1/1 I.U., Pluset) at 38.5°C and 5% CO_2, the oocytes were fixed in formalin, stained with a DAPI dye and fluorescently evaluated. None of the oocytes from the SSV group were matured *in vitro* and only 8.4% from the LV group showed the signs of nuclear maturation in contrast to 60.6% in the control (fresh) group. In all frozen ovarian fragments, regardless of vitrification technique, serious damages of oocytes at the light or electron microscopy levels were detected like extensive vacuolization and disintegration of the ooplasm and organelle dislocation. Visible zona pellucida cracks and deformities of oocytes were caused likely by the mechanical action of ice crystals formed in the follicle cavity. The granulosa cell nuclei were largely pyknotic. Germinal vesicles showed disintegrated nuclear envelope. Microvilli of cytoplasmic membrane were disrupted. The damaged *zona pellucida* acquired layer-like structure, and cells of *corona radiata* showed extensive damages. In conclusion, our experiments did not confirm that the oocytes, frozen in small antral follicles from ovarian tissue fragments using SSV or LV, are able to mature *in vitro,* due to extensive cellular damages revealed by histological and ultrastructural analyses.
Grant support: the Agency for the support of Science and Technology, Slovak Republic (APVV-15-0196).

A282E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

MitoQ rescues early embryo development of metabolically-compromised bovine oocytes *in vitro*

W.F.A. Marei, L. Van den Bosch, P.E.J. Bols, J.L.M.R. Leroy

Gamete Research Centre, University of Antwerp, Wilrijk, Belgium.

Keywords: MitoQ, embryos.

Maternal metabolic disorders e.g. obesity have been linked with reduced oocyte quality and subfertility. In these cases, upregulated lipolysis increases free fatty acid concentrations, predominantly palmitic acid (PA), in blood and ovarian follicular fluid, causing direct detrimental effects on oocyte quality and IVF results. Using proteomic analyses, we found that high cellular oxidative stress, mitochondrial dysfunction and unfolded protein responses (UPR) are key mechanisms explaining PA-linked lipotoxicity in bovine oocytes. Carryover of cellular stress to subsequent early embryonic stages increases the risk of embryo cell death and explains the reduced blastocyst rates observed *in vitro*. Mitochondria-targeted therapeutics are increasingly used to treat metabolic diseases, however, their efficiency in rescuing development of metabolically-compromised oocytes has not been examined. In the present study, oocytes matured in media containing high pathophysiological PA concentration (150μM) or solvent (Control) were cultured in the presence or absence of mitoquinone (MitoQ; a mitochondria-targeted antioxidant, 1μM) (from day 1 post-fertilization (D1) until D8, in serum-free FA-free SOF media). Embryo cleavage and fragmentation (at D2) and blastocyst rates (D8) were recorded. Gene expression patterns of markers of cellular stress were examined in the resultant D8 blastocysts. Numerical data were analyzed by ANOVA, and categorical data by binary logistic regression, followed by Bonferroni correction. At D2, PA had no effect ($P > 0.1$) on embryo cleavage (63.6 vs. 70.2%) and fragmentation (14.2 vs. 12.9%) rates compared to control. However, PA significantly reduced blastocyst rate at D8 (16.9 vs. 25.7%, $P < 0.05$). MitoQ supplementation during culture to PA-derived embryos significantly reduced embryo fragmentation (7.3 vs. 14.2%) and rescued embryo development to the blastocyst stage (25.0 vs. 16.9%) compared with PA-group cultured in the absence of MitoQ. Day 8 blastocysts derived from the PA group had significantly ($P < 0.05$) higher mRNA expression of genes related to oxidative stress (*CAT*, 5.5 ± 3.1 folds; *SOD2*, 3.4 ± 0.4; but not *GPx*, 1.99 ± 1.03); mitochondrial UPR (*HSPE1*, 4.2 ± 1.6 folds, and *HSPD1*, 10.6 ± 5.8 folds); and ER stress (*ATF4*, 2.2 ± 0.29 folds; *ATF6*, 25.99 ± 12.5 folds; and *HSPA5*, 10.7 ± 4.7 folds). In contrast, MitoQ supplementation to PA-embryos during culture significantly reduced the expression of the affected genes to control levels ($P > 0.05$). For oocytes matured in control conditions, MitoQ supplementation during culture had no significant effects on development and gene expression patterns ($P > 0.1$). In conclusion, these results provide further evidence that mitochondria play a central role in the pathogenesis of reduced oocyte developmental capacity under metabolic stress. Mitochondrial-targeted therapy (MitoQ) during early development of embryos derived from metabolically-compromised oocytes may be an efficient tool to reduce cellular stress level and maximize embryo development to the blastocyst stage. Further investigations are in progress to examine the quality of the rescued embryos.

A283E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

mtDNA content of bovine cumulus cells derived from oocytes with different developmental competence following individual culture

A. Martínez-Moro[1,2], I. Lamas-Toranzo[1], P. Bermejo-Álvarez[1]

[1]INIA, Madrid, Madrid, Spain; [2]Procreatec, Madrid, Madrid, Spain.

Keywords: Mitochondria, mtDNA, oocyte competence.

Mitochondria are the most abundant organelles in the mammalian oocyte and early embryo, where they serve relevant metabolic and signal transduction functions. Oocyte´s mitocondrial DNA (mtDNA) content increases during folliculogenesis and it has been positively linked with fertilization outcomes following IVF in humans, bovine and pigs. Cumulus cells are closely associated with the oocyte they nourish and may constitute a valuable biological material to perform non-invasive assays of oocyte´s developmental competence. The objective of this study has been to determine whether oocytes exhibiting a diverse developmental competence following individual *in vitro* maturation (iIVM), fertilization (iIVF) and culture (iIVC) exhibit differences in the mtDNA content of their cumulus cells. Cumulus-oocyte complexes (COCs) were obtained from slaughterhouse ovaries aspirating 2 to 8 mm follicles. Following conventional morphological COCs selection, iIVM was performed in 10 µl drops of maturation medium (TCM199 supplemented with 10 ng/ml EGF, 10% serum and gentamicin) covered by mineral oil. Following maturation, cumulus cells were removed by pipetting in a 0.1% hyaluronidase solution in PBS. Cumulus cells were pelleted by centrifugation at 1500 g for 5 min and pellet was snap frozen and stored at -80 ºC until analysis. Denuded oocytes were individually fertilized and cultured in 40 and 10 µl drops, respectively, covered under mineral oil. For mtDNA analysis cumulus cells samples were sorted in three groups based on the developmental competence of their corresponding oocyte following iIVF and iVC: 1) uncleaved (n = 15), 2) cleaved but not reaching the blastocyst stage (n = 15), and 3) developed to blastocyst (n = 11). Cumulus cells DNA was extracted by Picopure and mtDNA was quantified by qPCR using specific primers for the mitocondrial gene *ND1* and the autosomal gene *RN18S1*, used to normalize mtDNA copy number to the number of cells present in the sample. Relative mtDNA abundance did not differ between the different groups analyzed (1.08 ± 0.17 vs. 1 ± 0.14 vs. 1.12 ± 1.7 for oocytes resulting in not cleavage, cleavage arrest or blastocysts, respectively: mean ± s.e.m., ANOVA $p > 0.05$), suggesting that cumulus cells mtDNA is not predicitive of oocyte developmental ability under these conditions.

This work and AMM have been supported by the project IND2017/BIO-7748 by Madrid Region Government. ILT is supported by a FPI contract and PBA by a Ramón y Cajal Contract from the Spanish Ministry of Economy and Competitiveness.

A284E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

Good pregnancy rate of bovine biopsied and vitrified IVP embryos

E. Mullaart, F. Dotinga, H. Flapper, H. Knijn, R. Schaap

CRV, Arnhem, The Netherlands.

Keywords: IVP, biopsy, pregnancy.

Genomic selection is now routinely used to select the best animals for breeding. Basically the same method can also be used to select the best embryo. Since the process of genomic selection (i.e. genotyping and breeding value estimation) takes a few days, the embryo need to be frozen. Good pregnancy results are already obtained using biopsied flushed embryos after slow freezing with Ethylene Glycol (Ponsart Reprod Fertil Dev., 26, 12-21, 2013). However, when using IVP embryos, pregnancy rates are too low for commercial use (30% compared to 50%). Here we tested if vitrification of IVP embryos after biopsy give pregnancy results that are good enough to use the method in a commercial setting. IVP embryos are made from OPU derived COCs using standard production method of our company CRV (Merton, Reprod Domest Anim. 47, 1037-42, 2012). At day 7 quality 1 and 2 embryos are biopsied using the blade biopsy method. The DNA in the biopsy is pre-amplified and used for genotyping. The remaining embryo is vitrified using a mixture of Ethylene Glycol, DMSO and Ficoll. The CVM Vitrification Block and CVM Fibreplugs™ with sleeves were used as vitrification method. The embryos are placed for 2 minutes in a 7,5% EG/DMSO solution and 40 sec in a 15% EG/DMSO/Ficoll solution. Subsequently, the embryo is transferred on the nylon hook of the fibreplug in a total volume of 1,5µl and plunged into the LN and stored. Before transfer the embryos are thawed in a 3 step washing method using base medium containing Fetal Calf Serum and a decreasing percentage of sucrose. Embryos (53 quality 1 and 57 quality 2) were transported at a temperature of 25ºC and subsequently transferred to recipient animals on 5 different farms. Pregnancy rates are scored at 3 months after transfer of the embryo by ultrasound scanning. The results indicate that good pregnancy results are obtained from both quality 1 (55%) and quality 2 (44%) embryos. These pregnancy data are not different from those obtained with non-biopsied embryos (results not shown). As expected quality 1 embryos seem to give a higher pregnancy rate compared to quality 1, but numbers are yet too low to obtain a statistical significant difference.

We conclude that using vitrification as freezing method for biopsied IVP embryos, good pregnancy rates can be obtained (especially for quality 1) and can be used in a commercial setting.

A285E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

Peri-natal basic blood biochemistry and health of calves born from frozen and vitrified IVP cattle embryos

A.V. Murillo, M. Muñoz, D. Martin-Gonzalez, S. Carrocera, E. Gomez

SERIDA, Centro de Biotecnología Animal, Gijón, Asturias, Spain.

Techniques to cryopreserve bovine IVP embryos include vitrification/warming (V/W) and freezing/thawing (F/T). V and F differ in cryoprotectant concentration, cooling rates, and both may differently affect embryo physiology and survival. In this study we analyze whether calves born from V/W and F/T differ in metabolism and health status. Embryos were produced *in vitro* from slaughterhouse oocytes, fertilized with N = 3 bulls (2 Holstein and 1 Asturiana de los Valles –AV-) and cultured in SOFaaci as described (Gómez et al, Reprod Fertil Dev, 29:1932-43; 2017). Subsequently, expanded blastocysts were subjected to V/W (Gómez et al, 2017), or to F/T with an ethylene-glycol based method modified from Sanches et al, 2016 (Theriogenology, 85:1147-51). In experimental herd, N = 34 embryos were singly transferred to recipient heifers (Holstein, AV and their crosses), within replicates of N = 5-7 embryos in order to obtain age- and sire-matched calves. Gestation was allowed to end without calving induction. At birth, calves suckled colostrum from mothers ad-libitum. Clinical examinations and blood samples were taken 1-4 h after feeding (Day 0), and subsequently at fixed times (10 a.m. on days 15 and 30 of life). Blood was collected in vacuum tubes (lithium heparin) from jugular vein and directly analyzed in a Vetscan i-STAT One analyzer (Scil Animal Care, Madrid, Spain; CG4+ and Chem-8+ modules). Calves analyzed were N = 4 females and N = 4 males (V/W); and N = 5 males and N = 3 females (F/T; with 1 male dead on Day 17). Data were analyzed by ANOVA, and Bonferroni and REGWQ tests for FDR. The following parameters were affected ($P < 0.05$) by treatment (F/T vs V/W): Heart rate (149 ± 5 vs. 136 ± 5 beats/min); pCO_2 (39.4 ± 2.1 vs 44.6 ± 2.1 mmHg); packed cell volume (PCV; 22.6 ± 1.2 vs. 26.0 ± 1.2%PCV) and calculated hemoglobin (7.7 ± 0.4 vs. 8.8 ± 0.4). V/W tended to show higher values than F/T within HCO_3, sCO_2 and glucose ($P < 0.10$). Day x Treatment interactions ($P < 0.03$) were detected in T, Heart rate, respiratory rate, pCO_2, pO_2, base excess, HCO_3, sCO_2, sO_2, Na^+, glucose and creatinine. Day effects were identified between Day 0 vs. Day 15 and Day 30, whereby base excess, HCO_3, sCO_2, Na^+, creatinine, PCV and hemoglobin decreased; conversely, calf body weight, PO_2, sO_2 and Ca^{2+} increased within both F/T and V/W (P values between < 0.02 to < .0001). Calf sex affected Cl^-, PCV, hemoglobin and urea ($P < 0.05$), while appearance of conjunctival membrane, nasal secretions, K^+, lactate and anion gap were not affected. Interestingly, all values were comprised between normalcy intervals described in previous calf studies. However, subtle epigenetic changes may underlie the differences observed between calves born from F/T and V/W embryos. Comprehensive metabolomics and genomics studies are in course.

Acknowledgements: MINECO (AGL2016-78597-R) and FEDER; AM: SENESCYT-Ecuador-II Fase 2013. COST Action 16119, *In vitro* 3-D total cell guidance and fitness (Cellfit). Principado de Asturias, Plan de Ciencia, Tecnología e Innovación 2013-2017 (GRUPIN 14-114).

A286E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

Identification and mathematical prediction of different morphokinetic profiles of *in vitro* developed bovine embryos

A.P. Reis[1], M. Beghiti[1,3], S. Messoudi[3], B. Marquant-Le Guienne[2], L. Laffont[1], S. Ruffini[1], E. Canon[1], P. Adenot[1], N. Le Brusq[1], V. Duranthon[1], A. Trubuil[3]

[1]UMR BDR, INRA, ENVA, Université Paris Saclay, Jouy en Josas, France; [2]Allice, Recherche et Développement, Jouy en Josas, France; [3]MaIAGE, INRA, Jouy en Josas, France.

Keywords: embryo, morphokinetics, prediction.

The current method of embryo classification (IETS, 2013) is based on a static observation of *in vivo* derived embryos at day 7 post insemination (7 dpi). *In vitro* produced embryos (PIV) features impair their classification with this method. Morphokinetics is a powerful source of information to improve the comprehension of PIV embryo developmental behaviour. The objective of this study is to develop a methodology to read and predict different *in vitro* developmental potential of bovine PIV embryos by combinating morphokinetical parameters. Holstein embryos produced from oocytes recovered from slaughterhouse ovaries, *in vitro* fertilized with the semen of 4 different bulls and cultured for 8 days post insemination (8 dpi). Time lapse pictures were taken every 15 minutes throughout the culture period (672 pictures/embryo; Primovision™). The work was performed in 4 tasks (T): T1) identification of the profiles of *in vitro* development; T2) identification and standardisation of a reading method for bovine embryos morphokinetical parameters; T3) mathematical selection of a parsimonious subset of non-correlated parameters and construction of a predictor through the application of a supervised learning approach combining regression and classification (Random Forest) and creation of a mathematical predictor of the embryo development profiles. A total of 172 embryos were observed. T1: 6 morphokinetical profiles were retained: Arrested Embryos (AE: embryos without mitotic activity, showing signs of life); Dead Embryos (DE: embryos with all cells dead); Anarchic Embryos (ANE: embryos with abnormal morphological and/or kinetical development: some of these embryos can result in a blastocyst); Not Hatched Blastocysts (NHB: blastocysts not hatching by 8 dpi); Hatching Blastocysts (HB - blastocysts hatching *in vitro* from 7.3 dpi to 8 dpi) and Early Hatching Blastocysts (EHB - blastocysts hatching from 6 to 7.2 dpi). T2: a guideline was built to standardise reading of 116 parameters (i.e.: type, timing and duration of cell divisions and embryo cycles, LAG phase, cell degeneration, cytoplasmic particles, fragments, vacuoles,...); T3: a subset of parameters was selected and the mathematical predictor was built. The standardisation of the reading methodology is important to promote scientific exchange and study comparisons on the subject (to our knowledge this work resulted into the first morphokinetics reading guideline for the bovine PIV embryos). In addition, this initial work highlighted a new concept for the *in vitro* bovine embryo assessment and further valorisation: it takes into account the very early embryo's dynamic behaviour to predict its further potential of development. The robustness of the algorythm is satisfactory. The specificity, sensitivity, PPV, NPV of the predictive algorithm range respectively in the intervals [0,944; 0,977], [0,640; 0,962], [0,724; 0,892], [0,931; 0,992] for the different profiles. This predictive method can be useful in the field to select embryos for transfer and for research (groups of embryos sharing potential and morphokinetic similarities).

A287E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

Enriched n-3 polyunsaturated fatty acid diet modified oocyte lipid composition and may influence oocyte quality in Prim Holstein dairy cows

S. Uzbekova[1], P. Bertevello[1], O. Ghazouani[1], A.P. Teixeira-Gomes[3], A. Seyer[2], V. Maillard[1], M. Oseikria[1], C. Banliat[1], V. Labas[3], S. Elis[1]

[1]INRA PRC, Nouzilly, FRANCE; [2]Profilomic SA, Boulogne-Billancourt, France; [3]INRA CIRE PAIB, Nouzilly, France.

Keywords: n-3 PUFA, oocyte lipids, dairy cows.

Administration of long chain n-3 polyunsaturated fatty acid (n-3 PUFA) diet to dairy cows may impact oocyte quality (Elis et al, Animal Reproduction Science 164:121, 2016). Addition of docosahexaenoic acid (C22:6 n-3) during IVM led to higher blastocyst rate after IVF (Oseikria et al, Theriogenology 85(9):1625, 2016) and significantly changed oocyte lipid content (Elis et al, J Ovarian Res 10(1):74, 2017). The present objective was to compare lipid content of the oocytes from the dairy cows supplemented with n-3 or n-6 PUFA-enriched diet. Oocyte-cumulus complexes were aspirated by OPU after hormonal ovarian stimulation, from 18 primiparous Holstein dairy cows after 3 or 9 weeks of supplementation with 1% dry matter of either n-3 PUFAs (n = 9, micro encapsulated fish oil, OMG750®) or n-6 PUFA (n = 9, micro encapsulated soy oil, OMG Soy®) (Kemin). N-3 PUFA level in plasma and follicular fluid was measured after 2, 5 and 7 weeks of supplementation. Immature oocytes from n-3 and n-6 diet groups (60 and 61 oocytes, respectively) were denuded from CC and analyzed individually using an UltrafleXtreme MALDI-TOF/TOF instrument in positive reflector mode, with DHAP matrix. Lipid spectral profiles (3000 shots per spectra) were acquired for each oocyte. M/z peaks were detected in the range of 160 to 1000 m/z. Values of the normalized peak heights (NPH) were quantified and compared between the two groups by t-test with Benjamini-Hochberg correction. Multivariate Principal Component Analysis (PCA) was performed using differential NPHs. Lipids were identified by high-resolution mass spectrometry LC-MS or by direct infusion combined to top-down MS/MS analyses, and annotated according to Lipid maps database. Concentration of eicosapentaenoic acid (C20:5 n-3) and total n-3 PUFA significantly increased in n-3 group, after 2 weeks of diet in plasma, and after 5 weeks in follicular fluid, as compared to n-6 group. Body weight and milk production did not differ. Lipid profiles of the oocytes showed significant difference between n-3 and n-6 diets (97 up-regulated and 91 down-regulated peaks, $P < 0.05$, fold change > 2). PCA allowed clear discrimination of n-3 and n-6 groups. 40 differential peaks were identified (496-827 m/z); among them 12 phosphatidylcholines (PC), 3 phosphatidylethanolamines (PE, C36), 2 sphingomyelins (SM, C35) and lyso-phosphatidylcholine LPC 22:4 were more abundant in n-3 oocytes, whereas 14 PC, PE 30:0, SM 34:1, two LPC (16:0 and 18:0) and two triglycerides (46:1, 47:1) were more abundant in n-6 group. These variations indicated profound changes in composition of several lipid classes from oocyte membrane and intracellular pool, occurring after only few weeks of n-3 or n-6 PUFA dietary supplement. These cellular lipid changes may influence oocyte capacity to develop better blastocysts after IVF in n-3 supplemented cows (see Elis et al, AETE 2018), and highlight the importance of identifying beneficial oocyte lipid profile to improve embryo biotechnologies issues.

Bovomega3 project was funded by Val de Loire Region, France.

A288E Support biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology, and "omics"

Effect of seminal plasma proteins from stallions of proven fertility on frozen epididymal sperm

L.A. Vieira[1,2], C. Matas[1,2], J. Gadea[1,2]

[1]Department of Physiology, International Excellence Campus for Higher Education and Research, University of Murcia, Spain, Murcia, Spain; [2]Institute for Biomedical Research of Murcia IMIB-Arrixaca, Murcia, Spain, Murcia, Spain.

Keywords: seminal plasma, stallion, epididymal sperm.

The incubation of epididymal sperm with stallion seminal plasma could help to identify the components of the seminal plasma responsible for a beneficial effect or not on cryopreservation process of sperm. Our previous study showed that the preincubation of epididymal sperm with seminal plasma obtained from three different stallions (A, F, O) affected post-thaw sperm parameters differently. While two of them (F, O) improved post-thaw sperm viability and acrosome status of epididymal sperm, the other one (A) reduced these parameters compared to control. The aim was to determine the components of seminal plasma that could be involved in the improvement of the sperm parameters. Seminal samples from these stallions were evaluated using 2D-DIGE and liquid chromatography-mass spectrometry (LC-MS/MS) to discover the proteins involved in protecting spermatozoa during freezing. To our knowledge, this is the first report in the literature that compare the seminal plasma protein composition of several stallions. Seminal plasma was labeled using the kit AmershamCyDye DIGE Fluors (General Electric Healthcare España S. A., Madrid, Spain, ref. 25-8010-65) for two dimensional-fluorescence difference gel electrophoresis (2D DIGE). In each 2D gel, two samples were compared. Cy3 and Cy5 fluors were used for labeling each sample (A, F and O) separately, whereas Cy2 fluor labeled a mixture of both (A, F and O) and was used as normalization reference. Data were analyzed by one-way ANOVA followed by Dunnett´s multiple comparison tests ($P < 0.05$). Eight differentially expressed proteins were identified in seminal plasma samples. Four of them showed a higher expression in F and O samples which improved the frozen-thawed epididymal sperm parameters: 1) Cysteine-rich secreted protein 3; 2) Seminal plasma protein A3-like that belongs to the same family of Cysteine-rich secreted protein 3; 3) Thrombospondin type-1 domain-containing protein 7B; and 4) Seminal plasma protein 1 ($P < 0.05$). On the other hand, the other four proteins showed a higher expression in sample A that reduced the seminal parameters: 5) Polymeric immunoglobulin receptor, 6) Protein serum albumin precursor, 7) Kallikrein-1E2 precursor and 8) Matrix-remodeling-associated protein 5 ($P < 0.05$). Our results showed that some proteins from F and O seminal plasma were previously related to freezability (Cysteine-rich secreted protein 3 and seminal plasma protein A3-like), whilst A seminal plasma proteins (Polymeric immunoglobulin receptor, Matrix-remodeling-associated protein 5 and Kallikrein-1E2 precursor) suggest some alteration in the male genital tract. However, further in-depth research is needed to know what proportion of proteins, related to freezing, should be present in seminal plasma to be considered as a good freezability.
Supported by Fundación Séneca, Saavedra Fajardo (20020/SF/16). MINECO-FEDER (AGL 2015-66341-R).