Animal Reproduction

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Cumulus cells transcript abundance as a proxy for bovine oocyte quality

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Keywords: Cumulus cells, gene expression, oocyte quality, RNAseq.

The efficiency of *in vitro* embryo production protocols in cattle is hampered by the reduced developmental competence of *in vitro* matured oocytes. Oocytes are enclosed by cumulus cells which serve essential nourishing and signalling functions during folliculogenesis. These cells provide an attractive matrix on which to perform analyses aimed to understand the molecular roots of oocyte competence. The objective of this study was to analyze the transcriptional differences between cumulus cells from oocytes exhibiting different developmental potentials by RNA-seq. Cumulus-oocyte complexes were obtained from slaughtered cattle and individually matured *in vitro*. Following IVM, cumulus cells were removed by hyaluronidase treatment, pelleted, snap frozen in liquid nitrogen and stored at -80 ºC until analysis. Cumulus-free oocytes were fertilized using semen from a single bull and cultured *in vitro* individually. Cumulus cells were allocated into three groups according to the developmental potential of the oocyte: 1) oocytes developing to blastocysts following IVF (Bl+Cl+), 2) oocytes cleaving following IVF but arresting development prior to the blastocyst stage (Bl-Cl+), and 3) oocytes not cleaving following IVF (Bl-Cl-). RNAseq was performed on 4 (Bl-Cl-) or 5 samples (Bl+Cl+ and Bl-Cl+) per group, using the Illumina platform with >30 M reads/sample. Each sample contained cumulus cells from 10 cumulus-oocyte complexes (COCs). Differential expression was analysed by DESeq2 software. RNAseq analysis revealed 1609, 1466 and 1420 differentially expressed genes (DEGs) for the comparisons Bl+Cl+ vs. Bl-Cl+, Bl+Cl+ vs. Bl-Cl- and Bl-Cl+ vs. Bl-Cl, respectively, using a raw p value <0.05. These DEGs were narrowed down to 77, 80 and 32 DEGs for the comparisons Bl+Cl+ vs. Bl-Cl+, Bl+Cl+ vs. Bl-Cl- and Bl-Cl+ vs. Bl-Cl-, respectively, when an adjusted p value <0.05 was used. From these subsets of DEGs, 49, 50 and 18 DEGs, respectively, exhibited a fold change greater than 1.5. Focussing on DEGs in cumulus cells obtained from oocytes developing to blastocysts, 10 DEGs were common to both comparisons (10/49 from Bl+Cl+ vs. Bl-Cl+, 10/50 from Bl+Cl+ vs. Bl-Cl-). These DEGs correspond to 6 downregulated genes (*HBE1, ITGA1, PAPPA, AKAP12, ITGA5 and SLC1A4), and 4 genes upregulated (GSTA1, PSMB8, FMOD and SFRP4)* in Bl+Cl+ compared to the other groups. In conclusion, cumulus cells transcript abundance could be used as a predictor of the developmental potential of their enclosed oocyte.

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Early embryo morphokinetic parameters to predict bovine *in vitro* blastocyst development

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Keywords: blastocyst, early cleavage, time-lapse.

Monitoring morpho-kinetics using time-lapse technology at initial cleavage stages may assist embryologists in selecting embryos with the greatest developmental potential. The objective of this study was to establish the best cut-point on blastomere division times based on the probability of detection statistics for bovine *in vitro* blastocyst development using time-lapse technology. Cumulus oocyte complexes, collected from the slaughterhouse, were matured and fertilized in groups of 60 using routine methods. After 21 h of sperm-oocyte coincubation, presumed zygotes were randomly selected for culture in 9- or 16-microwells, and their development was monitored using time-lapse technology (Primo Vision; Vitrolife®, Göteborg Sweden). Remaining zygotes were conventionally cultured in groups (n = 25) as a control. Time-lapse technology allowed to take pictures of the zygotes every 10 min. Morpho-kinetic data included time (hours) to reach the first (1-2 cell), second (3-4 cell), third (5-8 cell), and fourth (9-16 cell), cell division. A receiving operator characteristic (ROC) and the area under the curve (AUC) were built in to find the cut-points with the highest sensitivity (Se) and specificity (Sp) to predict day 8 blastocyst development using the morpho-kinetic data of blastomere division times. Generalized mixed-effects models were used to test the effect of the cleavage time based on the best cut-point on day 8 blastocyst development. The cut-point for the first blastomere division was 31.6 h and had a better combination of Se (60.4%) and Sp (72.8%) and higher ($P < 0.05$) AUC (68.7%) than the second (44.2 h; Se = 88.7 and Sp = 34%; AUC = 55.2%), third (45.6 h; Se = 81.1 and Sp = 36.7%; AUC =50.9%), or fourth (77 h; Se = 86.5 and Sp = 40.6%; AUC = 53.9%) blastomere division. In 15 replicates, control (n = 366) resulted in a higher day 8 blastocyst development (43.8 ± 2.8%) than time-lapse (n = 294; 30.7 ± 2.8%; $P = 0.0006$). The cleavage rate was similar (P > 0.05) between control (77.4 ± 2.3%) and time-lapse (71.4 ± 2.6%). From cleaved zygotes, slow-cleaving embryos (based on the first blastomere division) presented lower ($P < 0.001$) day 8 blastocyst development (25.4 ± 3.8%) in comparison to fast-cleaving (58.3 ± 5.3%) and control embryos (55.7 ± 3.1%). The day 8 blastocyst development was similar ($P > 0.05$) for fast-cleaving and control embryos. Other studies have already shown that early cleavage is associated with higher blastocyst development. However, the categorization of cleavage speeds of previous studies was mainly based on arbitrary cut-points. This study, for the first time, implemented a ROC curve and the AUC to establish cut-points of blastomere division to predict blastocyst development. The ROC curve and the AUC are considered as the golden standard for determining how a predictor (blastomere division time) is capable of distinguishing between classes (slow vs. fast). Moreover, we also developed cut-points for further blastomere divisions (second, third, and fourth) and discovered that the speed of the first division is the best predictor for blastocyst development.

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Differential expression of mRNAs and miRNAs in the sperm of bulls of contrasting fertility

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Keywords: bull, sperm, non-coding RNAs.

Bulls used in artificial insemination with apparently normal semen, as assessed under the microscope, can vary significantly in their field fertility. A range of more advanced flow cytometric assessments of i*n vitro* sperm function have failed to reliably predict the field fertility of bulls. At a molecular level, it is known that the sperm transcriptome contains a rich population of messenger RNAs (mRNAs), long non-coding RNAs (lncRNAs) and small noncoding RNAs (sncRNAs), such as microRNAs (miRNAs). Together, these various transcripts are fundamental for sperm function and for successful fertilisation and embryo development. The objective of this study was to characterize the transcriptomic profile of sperm from high (HF) and low fertility (LF) bulls at the mRNA and miRNA level, in order to identify differentially expressed transcripts with potential as novel markers of fertility. Holstein Friesian bulls were assigned to either the HF or LF group (n=10 per group) based on adjusted fertility scores calculated from a record of at least 500 inseminations (+4.1±0.15% for HF and -7.6±1.50% for LF; mean of 0%). Total RNA was extracted from a pool of frozen-thawed semen straws from 3 ejaculates per bull. Absence of contamination with gDNA and RNAs originating from somatic cells was confirmed by RT-PCR. Transcriptomic profiles were obtained by performing mRNA-seq and miRNA-seq, which generated data from an average of 67.4 and 14.8 million reads, respectively. Six mRNAs and 13 miRNAs, respectively, were found to be differentially expressed between HF and LF bulls. Notably, the highly abundant protamine 1 (PRM1) mRNA had a higher expression in LF than HF bulls (P<0.05). As PRM1 is involved in sperm chromatin condensation during spermatogenesis, this could suggest that LF bulls exhibit an abnormal sperm chromatin structure. The gene pathways targeted by the 13 differentially expressed miRNAs were related to embryonic development and gene expression regulation, suggesting that, collectively, these miRNAs may have an impact on early embryo development. This study has identified potential biomarkers that could be used for improving semen quality assessment and predicting bull fertility.

Funded by Science Foundation Ireland (16/IA/4474).

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JUNO coated beads to discriminate sperm from high or low fertility bulls

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Keywords: JUNO, sperm binding, cattle.

The molecular mechanisms involved in gamete interaction should be explored to develop new consistent methods to predict sperm fertility. Our knowledge of this process at the molecular level is relatively poor, particularly in mammals. JUNO is the only oocyte plasma membrane receptor described in mice and humans to be involved in gamete binding. It is believed that it is conserved amongst mammals, since JUNO orthologues have been found in all mammalian genomes sequenced to date (Bianchi et al., Nature 508(7497):483-7, 2014). Thus, we hypothesized that the molecules involved in fertilization could be useful in the evaluation of sperm fertilizing capacity. Therefore, we propose that a sperm-binding assay using beads conjugated with recombinant bovine JUNO (rbJUNO) protein mimicking the egg's shape could discriminate sperm with different fertilizing capacity.
rbJUNO was expressed in Chinese hamster ovary cells and its successful conjugation to the beads was confirmed by SDS-PAGE and western blot probed with commercial antibodies. Two sperm-binding experiments were performed. In Experiment 1 (4 replicates), groups of 50-60 B_{JUNO} and B_{Ctrl} (beads without protein) were co-incubated with bull sperm. In Experiment 2 (3 replicates), sperm from bulls categorized as low (LF) or high (HF) fertility based on field fertility data were used. Moreover, the selection of the bulls was narrowed down based on cleavage rate following IVF, being >80% and <40% for the HF and LF bulls, respectively. 50-60 B_{JUNO} were co-incubated with thawed sperm from 2 HF and 2 LF bulls. Motile sperm in both experiments were selected through a 45/90% gradient (final concentration of 200,000 sperm/mL) and incubated in Fert-TALP for 2 h at 39 ºC, in 5% CO_2 and 20% O_2. Then, beads were fixed with glutaraldehyde (0.5 % v/v) and stained with Hoechst 33342 (0.01 mM). The number of sperm per bead was recorded and results analyzed by one-way ANOVA. A P value <0.05 was considered statistically significant.
rbJUNO exhibited the expected molecular weight (30 kDa) and its conjugation to the beads was time-stable. In Experiment 1, the mean number of sperm bound to beads (S/B) was higher (P<0.001) for B_{JUNO} (13.39 ± 0.71; n=277) than B_{Ctrl} (5.69 ± 0.32; n= 257), confirming that sperm mainly bind to beads coated with its specific receptor and suggesting that the JUNO-IZUMO1 interaction is conserved in cattle. In Experiment 2, (S/B) was higher (P<0.05) in the HF bull group (19.20 ± 0.90; N=310) compared to LF bulls (13.60 ± 0.80; N=288).
In conclusion, these preliminary data suggest that this sperm-binding assay is a valuable tool to investigate the role of proteins involved in gamete interaction and that it is able to discriminate bulls of different fertilizing capacity. Future studies using the proposed model, could help understand the molecular aspects of the sperm-egg binding process in depth.
Supported by Fundación Seneca (20887/PI/18), Science Foundation Ireland (16/IA/4474) and EMBO Short-Term Fellowship.

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In vitro maturation with glutathione ethyl ester improves embryo development potential of vitrified bovine oocytes

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Keywords: oxidative stress, ROS, cryopreservation.

Despite the increasing interest and applications of oocyte cryopreservation, fertilization rates and embryo developmental competence of vitrified bovine oocytes are still low. Vitrification may induce oxidative stress in mammalian oocytes, which result from unbalanced amounts of reactive oxygen species and the depletion of ooplasmic levels of glutathione (GSH). Glutathione ethyl ester (GSH-OEt), a cell-permeable GSH donor, has been proved to increase GSH content and improve oocyte maturation rate, spindle morphology and fertilization rate in bovine *in vitro* matured oocytes (*Curnow* et al. Reprod. Fertil. Dev, 2010, 22, 597–605). We hypothesized that addition of GSH-OEt to the *in vitro* maturation (IVM) medium would increase the cryotolerance of bovine oocytes to vitrification by increasing embryo developmental competence. Viable COC's were *in vitro* matured in: 1) Control: IVM medium: TCM199 + 20% Fetal Calf Serum + 10 ng/mL epidermal growth factor and 50 µg/mL gentamycin (n=381); and 2) GSH-OEt: IVM medium supplemented with 5mM of GSH-OEt (n=307). After 22 h of IVM, half of the oocytes from each group were vitrified/warmed using the Cryotop method (Morató *et al*. Cryobiology, 2008, 57, 137–141) and allowed to recover in their respective IVM media for 2 additional hours (VIT (n=136) and VIT GSH-OEt groups (n=134), respectively). After 24 h of IVM, oocytes were inseminated and *in vitro* cultured. Cleavage rate, 16-cell stage and blastocyst yield were assessed at 48 h, 96 h and day 7 (D7) and day 8 (D8) post-insemination, respectively. After checking normal distribution of the data by using Shapiro Wilk test, a lineal Mixed-Effect followed by a pairwise comparison test (Tukey-adjustment) was performed to analyze differences in embryo development ($P<0.05$). Cleavage rate and D7 blastocyst yield were higher ($P<0.05$) for fresh non-vitrified oocytes (Control: 73.1±3.8% and 14.7±1.7%; GSH-OEt: 74.8±3.3% and 20.2±6.5%, respectively) than for vitrified oocytes (VIT: 42.8±6.9% and 4.1±1.3%; VIT GSH-OEt: 45.5±10.2% and 6.6±0.8%, respectively), regardless of GSH-OEt treatment. However, embryos derived from non-vitrified groups or VIT GSH-OEt group exhibited significantly higher percentages of 16-cell stage embryos and D8 blastocysts (Control: 53.7±8.8% and 23.1±5.2%; GSH-OEt: 45.6±2% and 28.6±8.1%; VIT GSH-OEt: 37.5±2.4% and 13.8±3.2%, respectively) when compared to vitrified oocytes (25.5±4.1% and 5.3±0.6%, respectively). Similar D8 blastocyst hatching ability was observed among non-vitrified oocytes (27.1±14.7% and 22.7±5.4%) and oocytes vitrified after IVM with GSH-OEt (9.5±9.5%) while none of the D8 blastocysts derived from vitrified oocytes was able to hatch. In conclusion, supplementation with GSH-OEt during IVM improved embryo development of vitrified-warmed bovine oocytes, probably by protecting the oocytes from the oxidative stress induced by the vitrification.

This study was supported by the Spanish Ministry of Science and Innovation (Project AGL2016-79802-P and grant 2017-FI-00451) and the Generalitat de Catalunya (Project No. 2017 SGR 1229).

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Effect of equilibration time on gene expression of vitrified/warmed *in vitro* produced bovine embryos using the VitTrans device

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Keywords: mRNA, apoptosis, oxidative stress.

For the successful application of vitrification technology to field conditions, the procedures used for warming and transfer of the cryopreserved *in vitro* produced (IVP) bovine embryos should be as simple as possible. The device VitTrans, designed by our group, enables warming/dilution of IVP embryos and their transfer directly to recipient females in field conditions (Morató and Mogas, Cryobiology, 68, 288, 2014). During vitrification, the exposure time of the embryo to the vitrification solution is commonly considered to be critical, and should be strictly controlled within 1 min. However, the exposure time to the equilibration solution is flexible, ranged from 2 min to 15 min. This study aimed to examine the effect of different equilibration times on gene expression of bovine blastocysts vitrified/warmed using the VitTrans device. IVP day 7 expanded blastocysts were exposed to equilibration solution (7.5% ethylene glycol (EG) + 7.5% dimethyl-sulfoxide (DMSO) in TCM199 + 20% Foetal Calf Serum) for 3 min (Short equilibration: SE) (n=55) or 12 min (Long equilibration: LE) (n=51). Blastocysts were then transferred to vitrification solution (15% EG + 15% DMSO + 0.5M sucrose in TCM199 + 20% Foetal Calf Serum), loaded onto the VitTrans device and plunged into liquid nitrogen. The entire process from embryo immersion in the vitrification solution to plunging was completed within 1 min. Fresh non-vitrified blastocysts (n = 56) were set as a control group. Warming was performed by injecting 0,3 mL of warming solution (0.5 M sucrose in TCM199 + 20% Foetal Calf Serum) at 45°C into the inner channel of the device. Blastocysts were then cultured in SOF medium at 38.5º C in a 5% CO_2, 5% O_2 humidified atmosphere. The relative mRNA abundance for *BAX, BCL-2, SOD1, AQP3, CX43* and *IFNτ* genes of vitrified/warmed viable blastocysts was assessed at 24 h post-warming using RT-PCR. Gene expression was analysed by Kruskall-Wallis test and followed by two-way ANOVA. Significance was set at P≤0.05. Analysis for gene expression revealed no differences in *BAX, AQP3, CX43* and *IFNτ* genes between blastocysts vitrified after SE or LE, whereas significantly higher abundance of *BCL-2* and *SOD1* transcripts was observed in blastocysts vitrified after SE when compared to blastocysts exposed to LE. A clear trend (p=0.07) towards higher *CX43* expression was present in blastocysts exposed to SE when compared to LE group. To conclude, this study showed that embryos exposed to equilibration solution for 3 min previously to vitrification/warming using the VitTrans methodology regulated relative expression level of anti-apoptotic (*BCL-2*) and antioxidant enzyme (*SOD1*) genes, which may be indicative of a better embryo competence. Further studies are warranted to determine if these observations are related with a higher pregnancy rate.

This study was supported by the Spanish Ministry of Science and Innovation (Project AGL2016-79802-P and grant BES-2017-081962) and Generalitat de Catalunya (Project 2017 SGR 1229).

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Health status and blood biochemistry of calves born from cryopreserved and fresh IVP embryos

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Keywords: cryopreservation, calf health, blood biochemistry.

Reproductive techniques can lead to developmental abnormality and phenotypic changes in the offspring (Guo XY et al, Fertil Steril, 2017). In cattle, changes in calf phenotype due to embryo vitrification/warming (V/W) and freezing/thawing (F/T) are not well known. In this study we compared basic blood parameters and health of calves born from embryos transferred after V/W and F/T vs. fresh.
Abattoir oocytes were matured and fertilized *in vitro* with N=4 Asturiana de los Valles (AV) and N=3 Holstein bulls. Embryos were cultured in SOFaaci-BSA (with or w/o 0.1% FCS) from Day-0 to Day-6, and then w/o protein. Expanded blastocysts (Day-7; N=112) were singly transferred to recipient heifers (Holstein, AV and their crosses). Calves born were clinically examined and blood samples were taken before and 1 to 4h after colostrum intake. Blood was analysed in situ in a Vetscan i-STAT One analyser (Scil Animal Care, Madrid, Spain; CG4+ and CHEM8+ cartridges). Data were analysed by parametric (GLM; P<0.05) and non-parametric (Krustal-Wallis) tests (P<0.05), with REGWQ as a post-hoc test. Calves born after F/T (N=22), V/W (N=14) and from fresh (N=12) embryos did not differ in birth weight (39.4±2.3, 39.8±2.4 and 40.9±2.5 kg, respectively from now on) and gestation length (283.1±1.6, 284.5±1.2 and 283.0±1.7 days). Within F/T, V/W and fresh embryos, respectively, capillary refill time (CRT; 3.63±0.16, 3.22±0.17 and 2.51±0.19 s), rectal temperature (39±0.11, 38.9±0.12 and 38.3°C±0.12) and creatinine (3.8±0.21, 3.94±0.23 and 2.7±0.26 mg/dL) were higher (P<0.0004) after V/W and F/T. Partial pressure of CO_2 (PCO_2; 50.29±1.38, 53.21±1.47 and 56.6±1.68 mmHg) decreased (P<0.0151) after F/T, and packed cell volume (PCV; 23.01±1.07, 28.71±1.25 and 25.62±1.33 %PCV) and hemoglobin (g/dL) were higher (P<0.0002) after V/W. Colostrum intake decreased CRT (P<0.001), heart rate (beats/min; P<0.0282), pCO_2 (P<0.0001), creatinine (P<0.0125), PCV (P<0.0036) and hemoglobin (P<0.0034), while pH (P<0.0011) and base excess (mmol/L; P<0.0329) increased. All differences were subtle, as all parameters analyzed were comprised within healthy ranges. Further metabolomics studies are in course, in parallel to identification of putative epigenetic changes due to cryopreservation.
Acknowledgements: MINECO (AGL2016-78597-R and AGL2016-81890-REDT). FEDER. COST Action 16119 (Cellfit). ASEAVA. ASCOL. BES-2017-082200.

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The transcriptomic landscape of the rabbit oviduct through the pre-implantation embryo development

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Keywords: oviduct, transcriptomics, rabbit.

The mammalian oviduct is a specialized tubular structure surrounding the ovaries and connected with the uterus in which pre-implantational embryo development from fertilization up to the early blastocyst stage takes place. The understanding of the crosstalk between gametes or embryos and the oviduct is a challenge which we studied *in vivo* by the means of the omics in a polyovulatory and induced ovulator species such as the rabbit (*Oryctolagus cuniculus*). Twelve sexually mature New Zealand rabbit does received 0.02 mg Gonadorelin (im; Fertagyl, Merck., Kenilworth, USA) at insemination time (t=0 h) to induce ovulation. Isthmic tissue was collected after euthanasia at 10 h (pre-ovulatory; n=3), 20 h (post-ovulatory/zygote; n=3), 56 h (morula; n=3) and 88 h (blastocyst; n=3) post-insemination. RNA was extracted and samples with an RNA integrity number > 7 were analyzed by microarray GeneAtlas System (chip RabbitGene 1.1 ST, with 496321 probes covering 23282 genes, Affymetrix). Data analysis was done with R (v3.6), raw intensities were background corrected and RMA normalized. Differentially expressed genes (DEGs) were defined as those with a fold change > |2| and false discovery rate (FDR) < 0.05 obtained through a variance analysis and FDR adjustment with RankProd. All DEGs between the six possible comparisons were organized in clusters with SOTA (Self Organized Tree Algorithm). Biological meaning was assessed with Gene Ontology enrichment with DAVID (v6.8). The transition between the pre- and post-ovulatory stages involves 96 DEGs, the comparison between the post-ovulatory and the morula stage involves 122 DEGs, and the difference between the morula and blastocyst stages involves 48 DEGs. Interestingly, the transitions between the four stages involve meaningful changes in the expression of genes coding for secreted and exosomal proteins which could influence the local environment where embryos develop. Between the pre and post-ovulatory stages, 14 of these genes are downregulated and 10 upregulated. In the case of post-ovulatory to morula transition, 35 genes are downregulated and 24 upregulated. Furthermore, between the morula and blastocyst stages, 9 genes are downregulated. Genes involved in the extracellular matrix organization (e.g. collagens, and metalloproteases such as MMP7) account for most of these secreted protein coding genes downregulated between the pre-ovulatory to morula transition. Moreover, other important processes are associated with the zygote-morula transition: genes coding proteins involved in cell adhesion and lysil oxidases are downregulated, while chemoattractant protein coding genes are upregulated. To our knowledge, this is the first *in vivo* study of the transcriptomics of the oviduct in the presence of embryos in several stages of development. The findings reveal a series of changes in the transcriptomics of the oviduct which could be related to the communication of this organ with the pre-implantational embryo.
Supported by AGL2016-81890-REDT and PGC2018-094781-B-I00-MCIU/AEI/FEDER, UE.