A068E OPU/IVF/ET

Effects of an oil covered culture system on bovine *in vitro* produced embryos

Mariya Aravina, Sophie Diers, Christoph Knorr, Jens Tetens, Carina Blaschka

Division of Biotechnology and Livestock Reproduction, Department of Animal Sciences, Georg-August-University Goettingen, Germany.

Keywords: embryo, oil, cattle.

Embryos are usually produced in culture systems with an oil overlay, which conveys protection against the evaporation of water and microbial contamination. The oil can also release toxic substances and absorb essential components, such as hormones, which adversely affect the quality of the oocytes and the development of embryos in vitro. In addition, an oil overlay can be prohibitive when applying particular analysis such as concentration of lipophilic substances in medium, as steroids. The aim was to validate an oil-free bovine IVP system. This study compares bovine IVP with and without an oil overlay. Groups of 20 cumulus-oocyte-complexes (COC) collected from abattoir-derived ovaries were matured in tissue culture medium with BSA and eCG/hCG for 24 h with 5% CO2, fertilized in Fert-TALP for 19 h with 5% CO2 and cultured in SOFaa with 5% CO2 and 5% O2, all steps took place at 39°C. The quantity of medium in both groups (with and without an oil overlay) and throughout all stages of IVP was maintained at a volume of 100µl. The oil group was covered with 75µl paraffin oil (IVF Bioscience, Falmouth, UK). The maturation stage of oocytes was assessed using fluorescence staining (Hoechst 33342) after 24h of maturation. The developmental stage (number of blastocysts) were evaluated on day 8. The morphological quality of expanded day 8 blastocysts was determined by live-dead-staining (total cell number as well as ratio of live and dead cells). At least ten replicates were done. The statistical analysis was performed with ‘R’. Evaluation of maturation and development rates were analysed using a binomial test. Data obtained from the live-dead-staining were analysed using a t-test. Oocytes matured in the absence of an oil overlay had significantly ($p < 0.05$) higher maturation rates (71.5 ± 6.8%) when compared against matured in medium with an oil overlay (60.2 ± 9.3%). The developmental rate was significantly higher after culture without oil overlay (without oil: 38.4 ± 14.8%, with oil: 33.5 ± 12.6%; $p < 0.05$). The total cell number and the live-dead-ratio was not significantly ($p > 0.05$) different (total cell number: without oil: 130.0 ± 30.2, with oil: 119.0 ± 30.0; live-dead-ratio: without oil: 20.5 ± 11.5, with oil: 19.0 ± 8.0). The osmolarity did not differed between both groups during the IVP. Currently, the medium is analysed with regard to steroid concentrations via radioimmunoassay. So far, based on the higher maturation and development rates, bovine oil-free IVP-systems can be suggested as an alternative to oil covered medium, especially for maturation.

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A069 E OPU/IVF/ET

Individual serum-free and oil-based oocyte-to-embryo *in vitro* culture system is yielding high blastocyst rates and can be used as a basic system for individual follow-up

Nima Azari-Dolatabad[1], Annelies Raes[1], Anise Asaadi[1,2], Petra Van Damme[1], Ann Van Soom[1]

[1]Ghent University, Belgium; [2]Shiraz University.

Keywords: serum- free and individual culture system, embryo development, bovine.

Bovine *in vitro* embryo production (IVP) is routinely performed by culturing oocytes in group at ratio 1:2 (25/50µL droplets). We have recently shown that individual culture of bovine embryos in SOF-medium supplemented with 0.4 % BSA and insulin, transferrin and selenium (SOF-ITS-BSA) is yielding day 8-blastocyst rates over 40 %. However, in order to get these high blastocyst rates, *in vitro* maturation and fertilization still have to be performed in group culture. Several groups have attempted to develop an *in vitro* maturation-fertilization-culture system allowing individual follow-up from oocyte until embryo. Different approaches such as attaching the oocytes to the bottom of the Petri dish with Cell-Tak®, using a mesh grid or culturing oocytes and embryos in the well-of-the-well system have been attempted. These systems work well but are technically often challenging. Here we describe a simple individual oocyte-to-embryo culture system which is yielding routinely over 30 % blastocyst rates. *In vitro* maturation, fertilization and culture were either performed in group or in individual culture. For group culture, sixty cumulus-oocyte complexes were aspirated from ovaries derived from cows slaughtered in a local abattoir and matured in 500 µL TCM199 supplemented with 20 ng/mL EGF for 22h. Next, mature oocytes were incubated in 500 µL IVF-TALP with 1×10^6 spermatozoa/mL for 20h and then denuded and cultured in groups of 25 presumed zygotes in 50 µL droplets of SOF-ITS-BSA under paraffin oil (7.5 mL) overlay. For individual culture, 3 dishes (60×15 mm) with 20 µL droplets under paraffin oil overlay were used, each droplet containing one cumulus-oocyte-complex for maturation and subsequent fertilization (in the same media as described for group culture), and after denudation, presumed zygotes were cultured individually in 20 µL droplets SOF-ITS –BSA under paraffin oil overlay until day 8. Each dish contained 17 droplets. Blastocysts were then subjected to differential staining. Blastocyst rates (5 replicates) were significantly lower for individual compared to group culture (32 % (79/244) versus 47 % (146/314)) (Independent sample t-test, SPSS 20; $P<0.05$), but higher than 30 % so still acceptable. Blastocyst quality was also significantly lower, with a lower total cell number (90 ± 1.31 vs. 118 ± 1.16) and higher apoptotic cell ratio (8.4 ± 0.25% vs. 5.2 ± 0.19%) for individual versus group culture respectively. This indicates that despite the high overall blastocyst rates, there is still room for improvement in the individual culture system. In conclusion, the serum-free and oil-containing individual culture system we describe here is yielding acceptable blastocyst rates and can as such be used as to investigate (1) how differences in initial oocyte quality can affect embryo outcome; and (2) how addition of specific biochemical factors to the single oocyte maturation medium can be used in order to improve oocyte maturation. We are now testing the addition of different components derived from bovine follicular fluid to maturation medium in order to evaluate their possible effect on individual oocyte maturation and further embryo development.

A070E OPU/IVF/ET

Nobiletin supplementation prior to EGA improves development and quality of bovine blastocysts *in vitro*

Yulia N. Cajas[1], Karina E. Cañón-Beltrán[1], Claudia L.V. Leal[1,2], Encina González[3], Dimitrios Rizos[1]

[1]Department of Animal Reproduction, National Institute for Agriculture and Food Research and Technology (INIA), Spain; [2]Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, University of São Paulo, Pirassununga, Brazil; [3]Department of Anatomy and Embryology, Veterinary Faculty, Complutense University of Madrid (UCM), Madrid, Spain.

Keywords*:* Nobiletin, embryo, quality.

One of the most important events in early embryo development is the maternal-to-embryonic transition when maternal transcripts and proteins are gradually degraded and the embryonic genome is activated (EGA). In bovine embryos, major EGA occurs at the eight- to 16-cell stage. At the same time, an increase in ROS levels during embryo culture in vitro induces oxidative stress leading to failed embryonic development and low quality of the blastocysts produced. Nobiletin is a polymethoxyflavone with antioxidant properties in different cell types. Therefore, we aimed to evaluate the effect of nobiletin supplementation to the culture medium of bovine embryos before major EGA on their development and quality. In vitro produced zygotes were cultured in four-well plates with 500 µl SOF+5% FCS (control), control with 5, 10 or 25 µM nobiletin (MedChemExpress, MCE, Sweden) (Nob5, Nob10 and Nob25 respectively) or control with 0.03% dimethyl sulfoxide (CDMSO vehicle for nobiletin dilution) from 18 to 54 hours post-insemination (hpi) at 38.5ºC, 5% CO_2, 5% O_2 and 90% N_2. For all groups, the speed of development was considered and embryos that reached ≥8 -cells at 54 hpi were selected and cultured in control medium until Day 8. Cleavage rate (54 hpi) and blastocyst yield (D7-8) were evaluated, while quality of embryos were determined by assessing their total cell number, lipid content and mitochondrial activity (fluorescence intensity recorded in arbitrary units (a.u)). For this purpose, a representative number of D7 blastocysts (n=30/group/treatment) were fixed and stained with Hoëscht, Bodipy, and MitoTracker DeepRed, respectively. The images were obtained by confocal microscopy and analysed using Image J. Data obtained from 6 replicates were analysed using one-way ANOVA. No differences were found in cleavage rate while blastocyst yield at D8 was higher (P<0.001) for Nob5 (42.9±1.4%) and Nob10 (45.3±2.1%) compared to control (32.9±1.1%), CDMSO (32.6±1.4%) and Nob25 (34.2 ± 1.0%). For embryo quality evaluation, both controls and Nob groups with higher development were used. The number of intact cells per embryo was increased (P<0.001) in Nob5 (137.3±0.6) and Nob10 (126.7±0.8) compared to control (105.7±0.8) and CDMSO (106.4±0.8). The lipid content was significantly reduced (P<0.001) in Nob5 and Nob10 compared with both controls. For mitochondrial activity, fluorescence was significantly higher (P<0.001) in blastocysts from Nob5 and Nob10 compared with both controls. In conclusion, supplementation of nobiletin 5 or 10 µM/mL improves embryo development and the quality of blastocysts in terms of mitochondrial activity and cell numbers, while it reduces their lipid content.
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A071E OPU/IVF/ET

Effect of sperm selection using microfluidic sperm sorting chip on bovine embryonic development *in vitro*

Oguz Calisici[1], Adriana R. Camacho[1], Seckin Salar[2], Utkan Demirci[3], Árpád Csaba Bajcsy[1]

[1]University of Veterinary Medicine Hannover, Foundation, Germany; [2]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey; [3]Demirci Bio-Acoustic-MEMS in Medicine Labs (BAMM), Canary Center at Stanford for Cancer Early Detection, Department of Radiology, Stanford University School of Medicine University of Stanford, U.S.A.

Keywords*:* IVF, microfluidic sperm sorting chip, cattle.

The preparation of bovine sperm for in vitro fertilization (IVF) requires procedures such as a density-gradient centrifugation, which enables to select sperm cells with a higher rate of progressive motility and those that are morphologically intact. Microfluidic sorting devices have been demonstrated to effectively select motile human sperm without centrifugation. The aim of this study was to examine embryonic development after using a microfluidic sperm sorting chip for the selection of bovine sperm cells by the IVF procedure. Bovine ovaries were collected at slaughterhouse and placed and transported at 30°C in phosphate buffer solution until laboratory processing. In total 234 Cumulus-oocyte complexes (COCs) were obtained using the slicing method. The sperm samples were collected from a sire using an artificial vagina at artificial insemination centre. Sperm motility was assessed subjectively, and motile sperm (with more than 70% progressive motility) was prepared and used for IVF. After maturation, COCs (15-20 COCs in each drop) were fertilized with sperm cells that were selected after thawing at 30°C using either a standard density gradients (DG) protocol (SpermFilter®, GYNEMED GmbH & Co. KG, Lensahn, Germany) or a microfluidic sperm sorting (MSS) chip technique (Fertile Plus®, KOEK EU GmbH, Hannover, Germany). Motile sperm cells were added to the IVF drops to reach a final concentration of 1 × 106 cells/mL and were incubated with the COCs (19h, 5% CO2, 39°C). At the end of this co-incubation, presumptive zygotes were denuded using vortex, were washed and then placed into a synthetic oviduct fluid (SOF) under silicone oil. Cleavage and embryonic development rates (blastocyst formation) were recorded. For both treatment groups four replicates were performed. Statistical analysis was performed using Chi-square test with significance at $p < 0.05$. The MSS chip technique did not affect cleavage rates (MSS chip: 75.0% vs. DG: 71.2%, $p > 0.05$) and blastocyst rates at Day 7 (MSS chip: 18.1% vs. DG: 15.3%, $p > 0.05$). At Day 8, blastocysts rates were higher for oocytes, that were fertilized using MSS chip sorted sperms as compared to those selected by the DG technique (MSS chip: 33.6% vs. DG: 22.0%, $p = 0.048$). In conclusion, MSS chip sorted sperm may increase embryonic development rates and outcomes in routine IVF procedure. A larger number of sperm samples of different bulls will be studied in future studies to demonstrate the sperm quality and IVF outcomes after usage of MSS device.
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A072E OPU/IVF/ET

Melatonin increases the number of trophectoderm cells and total embryonic cells in *in vitro*-derived bovine blastocysts

Juan C. Gutiérrez-Añez[1,2], Patrick Aldag[1], Andrea Lucas-Hahn[1], Heiner Niemann[3]

[1]Department of Biotechnology, Friedrich Loeffler Institut, Mariensee, Lower Saxony, Germany; [2]Medical Surgical Department, College of Veterinary Medicine, University of Zulia, Maracaibo, Venezuela; [3]Hannover Medical School, Hannover, Hannover, Lower Saxony, Germany.

Keywords*:* Melatonin, inner cell mass, trophectoderm cells.

It has been shown that *in vitro*-derived embryos have fewer trophectodermal (TE) cells than *in vivo*-derived embryos. The TE cells are important for attachment of the embryo to the uterine endometrium, the formation of the fetal placenta and ultimately pregnancy establishment. An aberrant allocation of inner cell mass (ICM) and TE cells could be related to insufficient placentation and thus embryonic/fetal losses. Excess of oxidative stress under *in vitro* conditions can alter many important reactions affecting the embryonic development. Recently attention has been directed towards melatonin as a non-expensive broad-spectrum antioxidant. In the present study, we investigated the effects of melatonin on allocation of ICM and TE cells in *in vitro*-derived bovine embryos. A total of 97 blastocysts (Day 8) produced *in vitro* in the presence or absence of two concentrations of melatonin (MT) [MT 0.01nanomolar (nM): n=25 and MT 1.0 nM, n=21], were differentially stained to determine the number of cells (ICM and TE cells). As melatonin has to be dissolved in ethanol a "sham" group containing ethanol (ETOH; n=27) and a standard control group (Control: n=24) were also included in the experimental setting. A modified differential staining technique was applied (Thouas et al., Reprod Biomed Online 3(1): 25-29, 2001). Cells were counted via fluorescence microscopy (470-490 nm excitation filter) (Olympus BX60F, Tokyo, Japan) at 400-fold magnifications. The chromatin in nuclei of TE cells and ICM cells was stained and visualized by red/pink or blue color, respectively. Data were statistically analyzed using the SAS/STAT® software (version 9.3) with the general linear model (PROC GLM). Significant differences were defined at $p<0.05$. The general mean for TE, ICM, and total embryonic cells were 88.9±2.6, 41.9±1.3 and 130.8±3.2; respectively. The number of TE cells was significantly higher ($p<0.05$) in MT 0.01 nM and MT 1.0 nM groups compared to the control and ETH groups (101.3±11.8 and 101.6±8.6 vs. 86.5±12.2 and 83.6±12.2, respectively). No differences ($p>0.05$) were observed in the number of TE cells in sham controls and controls, as well as between both melatonin concentrations. There were no differences ($p>0.05$) regarding the number of ICM cells between the different experimental groups (Control: 43.8±6.9; ETOH: 39.0±6.9; MT 0.01 nM: 42.0±4.9 and MT 1nM: 46.1±6.7). Supplementation of the media with melatonin at 1.0 nM and 0.01 nM increased ($p=0.05$) total cell number compared with control and ethanol groups (147.3±14.6 and 143.7±10.7 vs. 130.3±15.1 and 122.5±15.1, respectively). No differences ($p>0.05$) were found between the control and the ETOH group, neither between both concentrations of melatonin. In conclusion, these data indicate that the presence of melatonin in in vitro embryo production media increases the allocation of embryonic to the trophectoderm, as well the total number of embryonic cells. The physiological importance of this finding warrants further study and could have an important implication to reduce early embryo/fetal losses observed after in vitro embryo production.

A073E OPU/IVF/ET

Perfluorooctane sulfonic acid (PFOS) affects early embryonic development in a bovine *in vitro* model

Ida Hallberg[1,7], Jonna Swahn[1], Sara Persson[1,2], Pauliina Damdimopoulou[3], Matts Olovsson[4], Joëlle Rüegg[5], Marc-André Sirard[6], Ylva Sjunnesson[1,7]

[1]Department of Clinical Science, Swedish University of Agricultural Science, Uppsala, Sweden; [2]Swedish Museum of Natural History, Department of Environmental research and monitoring, Stockholm, Sweden; [3]Department of Clinical Science, Intervention and Technology, Karolinska Institutet, Stockholm, Sweden; [4]Department of Women's and Children's Health, Uppsala University, Uppsala, Sweden; [5]Institute for Environmental Medicine, Karolinska Institutet, Stockholm, Sweden; [6]Department of Animal Sciences, Laval University, Quebec, Canada; [7]Centre for Reproductive Biology in Uppsala, University of Agricultural Sciences and Uppsala University, Uppsala, Sweden.

Keywords: Oocyte maturation, cattle, in vitro embryo production, PFOS.

Perfluorooctane sulfonic acid (PFOS) is a chemical that has been widely used in products like food packaging, textile, impregnations and firefighting foams. It is now banned in many countries, including the EU, but still present in nature, animals and humans due to its persistent and bioaccumulating properties. The average reported human serum PFOS levels vary from 4-70nM (median 30 nM) in the literature (and higher in especially exposed groups). PFOS is endocrine disruptive and has toxic effects on reproduction in research animals although human data remains contradictory. The aim of this study was to examine the effect of environmentally relevant concentrations of PFOS on bovine early embryo development *in vitro* as a possible model for human early embryo development. This model was chosen as the process of maturation and fertilization in bovine oocytes shows more similarities to the human process compared to the murine. Abattoir derived oocytes were matured, fertilized and cultured in a bovine *in vitro* system. Oocytes were randomly divided into two treatment groups exposed to either 20 nM PFOS (P20) or 200 nM PFOS (P200) during *in vitro* maturation and to one control group (C) without PFOS. Cleavage rate as well as stage and grade of day 7 and 8 blastocysts were assessed. Further, neutral lipids were analyzed using HCS LipidTOXTM Green Neutral Lipid Stain (ThermoFisher Scientific, Waltham, USA) and nuclei were stained with Hoechst 33342 in paraformaldehyde-fixed day 8 blastocysts. Evaluations of the number of nuclei, total lipid volume, lipid volume of each blastocyst and lipid droplet size were performed using confocal microscopy. Mixed effect logistic regression was used to calculate the effect of treatment on the number of cleaved embryos and developed blastocysts. The effect of treatment on lipid droplets was performed using a linear mixed effect model with log-transformed values to assume normal distribution. From 13 batches with a total of 847 oocytes, 162 blastocysts were developed. Cleavage rate and cleavage rate above the 2-cell stage were significantly lower in the P200 group compared to the control group, although no significant difference could be seen on blastocyst development or grade on day 7 and 8 neither in P20 or P200. The blastocyst stage of development was significantly lower in the P200 group compared to the C group. In addition, in the P200 group PFOS had an effect on lipid droplet size in the early blastocyst stage where the lipid droplets were larger. The results from this experiment indicate that human relevant concentrations of PFOS impair bovine early embryo development. PFOS exposure delays development and affects the size of lipid droplets. These findings are in line with epidemiological studies linking PFOS exposure to lipid metabolism in adults. More studies are needed to elucidate the mechanisms and effects of PFOS on the early embryo development.
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A074E OPU/IVF/ET

Quality of mouse IVF blastocysts after addition of quercetin to the culture media at the morula stage

Nuria Hernández Rollán[1], Marta López Morató[1], José Mijares Gordún[1], Soledad Sánchez Mateos[1], Francisco Miguel Sánchez Margallo[1], Ignacio Santiago Álvarez Miguel[2]

[1]Jesús Usón Minimally Invasive Surgery Centre, Spain; [2]Cell Biology Department. Faculty of Medicine. University of Extremadura.

Keywords: IVF, culture-medium, blastocysts.

Quercetin is a plant flavonol found in many fruits and wine, which has an important role as antioxidant in many pathological pathways associated with oxidative stress. The effects of the quercetin have been studied on the *in vitro* maturation, the competency of the oocyte and during the whole embryo development in different animal species. Nonetheless its effects have not been studied on mouse embryos obtained by IVF only during the stage from morula to blastocyst. The aim of this study was to examine the embryo development, cell death and cell number on mouse blastocysts obtained by IVF and cultured under conditions of normoxy, hypoxy and with different concentrations of quercetin. B6D2 strain female mice were hormonally stimulated to activate the recruiting of the follicles and to trigger the ovulation. Mature cumulus-oocyte complexes were obtained, used to perform IVF, cultured in KSOM and divided into 6 groups when they reached the morula stage (day 3): IVF_{KSOM}: embryos were cultured in KSOM until the blastocyst stage (control group); $IVF_{50\mu M}$, $IVF_{10\mu M}$,$IVF_{5\mu M}$ and$IVF_{1\mu M}$: morulae were cultured for 4 hours in KSOM media supplemented with 50 µg/ml, 10 µg/ml, 5 µg/ml or 1 µg/ml of quercetin, respectively, in an atmosphere of 5% CO_2, and transferred back to normal KSOM and cultured until the stage of blastocyst; $IVF_{3\%}$: morulae were cultured for 4 hours in KSOM in an atmosphere of 3% of O_2 (to resemble the uterus condition after the morula stage) and 5% of CO_2 until the stage of blastocyst. The blastocysts were used to study the embryo development (n=15 IVFs/group), the total number of cells (trophoblast cells and ICM by DAPI staining, n=30 blastocysts/group) and the cell death (studied by TUNEL assay, n=25 blastocysts/group). Our results showed that the mean of embryos that developed to blastocyst was 59.90% $\pm$ 25.83 for IVF_{KSOM}, 61.04% $\pm$ 25.9 for $IVF_{50\mu M}$, 72.14% $\pm$ 22.69 for$IVF_{10\mu M}$, 62.27% $\pm$ 29.59 for $IVF_{5\mu M}$,68.57% $\pm$ 20.55 for $IVF_{1\mu M}$ and 63.76% $\pm$ 26.86 for $IVF_{3\%}$ ($p>0.05$). The mean of the number of cells per blastocyst was 84.1 $\pm$ 7.82 for IVF_{KSOM}, 82.23 $\pm$ 11.33 for $IVF_{50\mu M}$, 89 $\pm$ 15.6 for $IVF_{10\mu M}$, 86.7 $\pm$ 9.5 for $IVF_{5\mu M}$, 87.56 $\pm$ 10.99 for $IVF_{1\mu M}$ and 88.2 $\pm$ 12.89 for $IVF_{3\%}$ ($p>0.05$). Results for the cell death showed that the mean of dead cells per blastocyst was 0.10 $\pm$ 0.27 for IVF_{KSOM}, 0.12 $\pm$ 0.33 for $IVF_{50\mu M}$, 0.04 $\pm$ 0.2 for $IVF_{10\mu M}$, 0.08 $\pm$ 0.27 for $IVF_{5\mu M}$, 0.08 $\pm$ 0.27 for $IVF_{1\mu M}$ and 0.30 $\pm$ 0.45 for $IVF_{3\%}$ ($p>0.05$). One-way ANOVA test was used for the statistical analysis and a $p<0.05$ was considered statistically significant. Based on the p values, there were no statistically significant differences between the groups in any assay. It is worth mentioning though that in embryos cultured with 10 µM of quercetin the number of cells per blastocyst was higher, the cell death was lower and a higher number of embryos reached the blastocyst stage. These results show that, even though the differences found were not statistically significant, the enrichment of the embryo culture media with 10 µM of quercetin at the stage of morula slightly improves the mouse blastocyst quality, hence showing potential to increase the implantation rates. Further studies are required though to verify this hypothesis.

A075E OPU/IVF/ET

Hormone levels differ between cow recipients carrying *in vivo* or *in vitro*-derived conceptus during early pregnancy

Jordana S. Lopes[1,2], Estefanía Alcázar-Triviño[3], Cristina Soriano-Úbeda[1], Evelyne París-Oller[1,2], Pilar Coy[1,2]

[1]Department of Physiology, Faculty of Veterinary Medicine, University of Murcia - Campus Mare Nostrum, Murcia, Spain; [2]Institute for Biomedical Research of Murcia, IMIB-Arrixaca, Murcia, Spain; [3]El Barranquillo S.L., Torre Pacheco, Murcia, Spain.

Keywords*:* Embryo transfer, reproductive fluids, anti-mullerian hormone.

Bovine embryo production is still far from optimal. In order to better mimic the natural environment, natural reproductive fluids (RF), collected from reproductive tracts, have been proposed as additives for embryo culture and results from RF-derived embryos after transfer (ET) have not shown adverse effect on pregnancy rate at day 30 when compared to a control (BSA) (Lopes, Animal Reproduction, v15, nr3, p550, 2018). However, pregnancy maintenance is dependent on several factors and hormones such as progesterone (P4), estrogen (E2), cortisol or, more recently studied, anti-Mullerian hormone (AMH), play an important role. P4 and E2 are protagonists during pregnancy and P4 has been related to early growth of conceptus in uterus (Shorten, J Dairy Sci, 101:736-751, 2018). Cortisol, on the other hand has been tagged as a meaningful participant in intrauterine regulatory system of early pregnancy in cattle (Majewska, J Rep Imuno, 93:82-93, 2012). AMH levels are known to influence the pregnancy maintenance, being low levels associated with pregnancy loss (Ribeiro, J Dairy Sci, 97:6888–6900, 2014). Therefore, we hypothesised that P4, E2, cortisol and AMH levels might have an influence on recipient's pregnancy outcomes. To test our hypothesis bovine IVP-blastocyts cultured with or without RF (RF and BSA groups) were transferred to synchronized recipients. An in vivo control was added by artificial insemination (AI) of recipients with frozen-thawed semen from the same bull used to produce IVP embryos. At day 30, pregnancy diagnosis was performed, embryos measured and blood samples from pregnant recipients were taken (Pregnancy/ET or AI: 12/54 from RF, 10/45 from BSA and 8/35 from AI group). Blood collected on the day of ET and 7 days post-AI was also analysed. Statistical analysis consisted of Pearson's correlation between variables (group, hormones, embryo size, day) followed by one-way (groups) or t-test (day) when significant correlation ($p<0,05$) was found. At day 7, P4 was significantly lower in AI vs. both RF/BSA. Cortisol had a tendency to be lower for RF group ($p=0,052$) but was not confirmed by post-hoc. AMH and E2 were not different between groups. At day 30, AMH and E2 were lower and higher, respectively, between AI & BSA, but RF had intermediate concentrations. P4 and cortisol were not different between groups. No correlation was found between embryo' dimensions vs. hormone levels in any day nor group. P4 was, independently of the group, significantly lower at day 7 vs. day 30, whereas cortisol, E2 and AMH remained similar. In conclusion, in our study we observed that AMH and E2 levels at day 30 were significantly different between recipients holding AI vs. BSA embryos while recipients from RF group showed an intermediate value. P4 values, 7 days post-AI vs. day of ET were significantly lower for AI vs. ET recipients, but those differences disappeared by day 30. How the embryo influences hormonal levels remains to be further investigated and more analyses to the pregnant recipients as well as non-pregnant animals should be further addressed.

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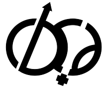

A076E OPU/IVF/ET

Use of in-estrus heifer serum on *in vitro* culture of sheep embryos

Alfredo Lorenzo[1], Raymundo Rangel[1], Rodolfo Ramírez[1], Demetrio Alonso Ambríz[2], José Ernesto Hernández[2]

[1]Universidad Autónoma Chapingo, Estado de México, México; [2]Universidad Autónoma Metropolitana, Ciudad de México, México.

Keywords: embryo culture, supplement, blastocyst.

Fetal bovine serum (FBS) is a universal supplement for embryo culture; however, alternatives have been sought to replace it. The study aimed to evaluate the estrus heifer serum (EHS) as a supplement of the culture medium, and to evaluate the differences between sera from donor Holstein heifers used individually or mixed in the development of ovine embryos to blastocyst stage cultured *in vitro*. A total of 1105 oocytes from ovaries of ewes obtained from a commercial slaughterhouse were used. The oocytes were *in vitro* matured (IVM) in TCM 199 (In vitro S.A., Mexico), supplemented with: 10% of FBS (Biowest, Mayimex, Mexico), 5 µg mL^{-1} FSH (Folltropin-V, Bioniche, Canada), 5 IU mL^{-1} hCG (Chorulon, Intervet, Netherlands) and 1 µg mL^{-1} 17-β estradiol (E8875, Sigma, Mexico). After 24 h of IVM at 5% CO_2 in air, at 38.5 °C and saturated humidity, the oocytes were fertilized (IVF) in commercial medium (In vitro S.A., Mexico) using fresh spermatozoa ($1x10^6$ mL^{-1}) from a Rideau Arcott ram of known fertility. The zygotes were cultured using Cleavage medium (Cook IVF, Brisbane, Australia) for 72 h until 16-cell stage. The rate of IVM, IVF and embryos in the 16-cell stage were registered (85.5, 67.0 and 65.4%, respectively). The embryos in the 16-cell stage (723) were randomly assigned to one of five treatments with Blastocyst culture medium (Cook IVF, Brisbane, Australia) plus 10% serum of different types: T1: FBS (control, n= 146); T2: EHS1 (n= 144); T3: EHS2 (n= 143); T4: EHS3 (n= 143) and T5: EHS mixture (T2, T3 and T4) (n= 147). The embryos were cultured for 96 h until blastocyst stage. The development and quality of the blastocysts were evaluated according to their morphology, while the diameter blastocyst was measured with a digital camera (AmScope, MU1803, China) using an inverted microscope (Nikon, Eclipse TS100, Japan). These variables were analyzed using the SAS program. The development data consider a comparison of binomial proportions with the construction of confidence intervals using GENMOD. Analysis of variance was used to analyze the diameter results with a classification criteria and fixed effects using GLM, while blastocyst quality was modeled according to a multinomial distribution and analyzed with GENMOD procedure. The percentage of blastocysts was similar between treatments (41.8, 40.3, 39.9, 50.4 and 43.5% for T1, T2, T3, T4 and T5, respectively, p>0.05). For blastocyst diameter, T4 and T5 were larger than T1 (238 *vs* 223 µm, p= 0.007, 234 *vs* 223 µm, p=0.04, respectively). Likewise, T4 was larger than T2 (238 *vs* 226 µm, p=0.03). No differences were observed among treatments for blastocyst quality (p>0.05). In conclusion, the estrus heifer serum used has similar effects as fetal bovine serum when the culture medium is supplemented at 72 h. Moreover, the individual use of serums may be better as a supplement than using a mixture under the conditions of this study.

A077E OPU/IVF/ET

The efficiency of collecting *in vivo*-developed porcine zygotes is not affected by 3-to- 5-days weaning-to-estrus interval

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

[1]Dept. of Medicine and Animal Surgery, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum", University of Murcia, Murcia, Spain; [2]Dept. of Clinical & Experimental Medicine (IKE), Linköping University, Linköping, Sweden.

Keywords: zygote, pig, embryo collection.

The recently developed genome-editing (ZG-E) technology for pig zygotes, opening a new revolution in agriculture and biomedicine, depends of the efficient collection of large numbers of zygotes of the highest quality. Because IVP of zygotes in pigs still is inefficient, *in vivo*-collection remains as major source of zygotes. Little information is available on the efficiency of the collection procedures for in vivo-derived zygotes. Since the interval between pronuclear formation and the first division is very short in pigs, the collection of zygotes must be performed within a very narrow window. While the weaning is an efficient method to synchronize estrus and ovulation in sows, the weaning to estrus interval (WEI) can, due to its inverse relation with length of estrus and time of ovulation, interfere with ovulation and make it asynchronous. In addition, individual sows show variability in ovulation time, even after hCG treatment, which reduces the probability of obtaining zygotes during collection. This study compared the effects of three WEIs: 3d (N=57), 4d (N=131) or 5d (N=29) on the efficiency of zygote collection *in vivo*. The donors were super-ovulated with eCG (1,000 IU; i.m.) 24 h after weaning. Estrus was checked twice per day when allowing snout-to-snout contact of sows and a mature boar while applying manual backpressure. Sows in estrus at 48–72 h post-eCG were treated with hCG (750 IU; i.m.) at the onset of estrus. The donors were inseminated at 6 and 24 h after the onset of estrus and subjected to a laparotomy on Day 2 (Day 0: onset of estrus). After counting the number of corpora lutea, each oviduct was flushed with 20 mL of Tyrode's lactate-HEPES-polyvinyl alcohol medium. Collected structures were evaluated for morphology under a stereomicroscope and only those with a single cell and two visible polar bodies were considered as zygotes. Results were expressed as percentages or means±SD. Differences among groups were analyzed using Fisher's exact test or ANOVA as appropriate and were considered significant when $P<0.05$. A total of 217 out of 223 donors (97.3%) had embryos at collection. The mean ovulation rate (27.3±7.6 corpora lutea) and the mean number of structures (25.2±9.4) collected in these sows did not differ between groups. Of all recovered structures (N=5,468), 67.4%, 31.1% and 1.5% were zygotes, two-to-four cell embryos and oocytes-degenerated embryos, respectively. The different WEIs did neither affect the percentages of collected zygotes (range: 64.1% to 70.0%) nor the percentages of sows with zygotes at the collection (range: 70.2% to 73.3%). In conclusion, these results indicate that neither fertilization rates nor the number of zygotes collected at Day 2 of the cycle from superovulated sows were affected by a WEI of 3 to 5 days.

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A078E OPU/IVF/ET

Comparative study of growth parameters in piglets derived from embryos produced *in vitro* with or without reproductive fluids, and piglets derived from artificial insemination

E. París-Oller[1,3], R. Belda-Pérez[1], C. Soriano-Úbeda[1,3], L. Sarrías-Gil[1], A. Canha-Gouveia[1,3], R. Latorre[2], L.A. Vieira[1,3], O. Simonik[1], S. Ruíz[1,3], J. Gadea[1,3], C. Matás[1,3], P. Coy[1,3]

[1]Department of PhysiologyVeterinary Faculty, University of Murcia, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum", Murcia, Spain; [2]Department of Anatomy and Compared Pathology, Veterinary Faculty, University of Murcia, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum", Murcia, Spain; [3]Institute for Biomedical Research of Murcia (IMIB-Arrixaca), Murcia, Spain.

Keywords: Growth, Reproductive Fluids, pig.

Different studies have suggested that the use of assisted reproductive technologies (ART) is associated to a higher incidence of low birth weight (Castillo et al., Hum Reprod doi:10.1093/humrep/dez025, 2019) as well as to alterations in the growth curve (Donjacour et al., Biol Reprod 90:80, 1–10, 2014). Recently, it has been described that porcine blastocysts produced *in vitro* with reproductive fluids (RF) in the culture medium show DNA methylation and gene expression patterns more similar to those produced in vivo than their counterparts produced without RF (Cánovas et al, eLife 1: e23670, 2017). However, it is unknown whether the presence of RF during fertilization and embryo development *in vitro* affects offspring growth-related parameters such as weight and length. The objective of this work was to compare growth parameters of piglets born after the transfer of embryos produced *in vitro* with RF (F-IVP, N=19) or without RF (C-IVP, N=29) added to the culture media with the same parameters in animals derived from artificial insemination (AI, N= 57). After birth, piglets were weighed at different days (0, 3, 9, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180) with a mobile scale and average weight daily gain (AWG) was calculated from total weight values (W) at every two consecutive days. All animals were measured with a zoo-metric tape from the frontal region of the cranium to the beginning of the rump (Cranium-rump length, CRL). All weight data and female (N=42) CRL data were analyzed by robust mixed ANOVA test while mixed ANOVA test was used for male CRL data (N=32) because Mauchly's test for sphericity came out significant but Greenhouse-Geisser (GGe) and Huyhn-Feldt (HFe)corrections were not valid. A total of 4 litters per group were studied, with the litter sizes differing between them (4.75±1.71, 7.25±2.06, 14.25±6.55, for F-IVP, C-IVP, and AI, respectively). Significant differences were reported most days studied for W, AWG and CRL measurements ($P \leq 0.05$) when they were compared by genders in AI piglets versus F-IVP and C-IVP groups, being these last two heaviest and longest than the first, although F-IVP showed intermediate values that could be related to a phenotype more similar to that obtained through AI. Similarly, significant differences were also observed between F-IVP and C-IVP for W, AWG, and CRL from day 9 to day 75. However, due to the low number of piglets under study and to the high differences in litter sizes between groups, further analyses are necessary to elucidate the influence of co-variables such as the mentioned litter size, with a possible strong influence in growth rates. Though preliminary, these are the first data in a large animal model, up to our knowledge, comparing growth parameters between ART-derived and AI derived offspring. In addition, they shed light on possible future phenotypic differences between ART-derived animals produces with or without RF.
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A079E OPU/IVF/ET

Differences in glucose tolerance between piglets born after *in vitro* fertilization/ embryo transfer and relatives born after artificial insemination

E. París-Oller[1,3], S. Navarro-Serna[1,3], C. Soriano-Úbeda[1,3], J.S. Lopes[1,3], E. Rodríguez-Tobón[1], F.A. García-Vázquez[1,3], O. López-Albors[2], R. Romar[1,3], P. Coy[1,3], S. Cánovas[1,3]

[1]Department of Physiology, Veterinary Faculty, University of Murcia, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum", Murcia, Spain; [2]Department of Anatomy and Compared Pathology, Veterinary Faculty, University of Murcia, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum", Murcia, Spain; [3]Institute for Biomedical Research of Murcia (IMIB-Arrixaca), Murcia, Spain.

Keywords: glucose, reproductive fluids, ART.

Increasing evidence indicates a higher incidence of glucose metabolism abnormalities in children derived from Assisted Reproductive Technologies (ART) (Chen et al., Diabetes, 63:3189–3198,2014). Monophasic and biphasic patterns of blood glucose curves have been identified, biphasic curves being associated with better glucose tolerance and beta-cells function, increasing insulin sensitivity in humans (Bervoets et al., Horm Metab, 47:445–451, 2014). On the other hand, the addition of reproductive fluids (RF) to in vitro culture media used during ART has been proposed as a possible way to avoid ART-derived abnormalities in pigs (Cánovas et al., eLife 1: e23670, 2017). The aim of this study was to evaluate the response to oral glucose tolerance test in young growing pigs born from embryos produced in vitro with (F-IVP) or without RF (C-IVP), compared to animals born by artificial insemination (AI). Four litters of relatives animals per group were used. At 45 days of life, the two males and two females of highest and lowest weight per litter were selected for the study (N=14, N=15, and N=16 for F-IVP, C-IVP, and AI, respectively). After 18h overnight fast, water was withdrawn and 1h later, pigs drank 1.75 g/kg BW of glucose solution. Blood samples were collected from the auricular lateral vein before and 5, 10, 15, 20, 30, 45, 60, 90, 120 and 150 min after glucose intake. Blood glucose concentration (GC) was immediately determined by test strips with a glucometer (GlucoMenLX Plus+). One way ANOVA and Tukey post-hoc tests were applied ($P<0.05$). Pearson correlation coefficient was used to detect litter influence, resulting in a positive correlation between GC value and weight. A monophasic GC curve was observed in the three groups. GC steadily increased reaching a maximum at 45 min after glucose intake, thereafter, it decreased until basal values (range 74,13-78,67mg/dl). Significant differences between AI and F-IVP groups were observed at 15, 20 and 30 min, with F-IVP showing higher values. When the analyses were repeated splitting the animals by sex, males showed a monophasic curve with similar basal levels, and the glucose peak at 45 min in all groups (range 98,43-115,0mg/dl). Significant differences between AI and F-IVP groups were present at 20 min and between F-IVP vs.AI and C-IVP groups at 30 min. In contrast, females showed significant differences between AI and F-IVP groups before glucose intake and at 20 min, while F-IVP was different vs.AI and C-IVP at 15 min. In addition, females on average from F-IVP group presented a biphasic curve, with two peaks at 15 (range 83,50- 128,67mg/dl) and 45 min (range 100,88-128,20mg/dl) compared with other groups. In conclusion, glucose tolerance in growing piglets is affected by sex and by the origin of the embryo, although all the basal and peak values are always into the physiological range.

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A080E OPU/IVF/ET

Effect of type of recipient on an embryo transfer programme in sheep

Raymundo Rangel-Santos[1], Raymundo Rodríguez-De Lara[1], Demetrio A. Ambríz-García[2], María Del C. Navarro-Maldonado[2], Roberto Ramírez-López[3]

[1]Universidad Autónoma Chapingo. Chapingo Estado de México, México; [2]Universidad Autónoma Metropolitana. Ciudad de México, México; [3]Rancho Poza Rica, Singuilucan, Hidalgo, México.

Keywords: ewes, embryo transfer, pregnancy.

The implementation of embryo transfer is important to help in the multiplication of high genetic merit animals, through the selection of the best males and females. The success of the technique depends on the possibility of achieving high levels of efficiency in each step to get high rates of pregnancy. The objective of this study was to evaluate the fertility of two groups of ewes (hair and wool types) exposed to an embryo transfer programme. The study was conducted from September to December of 2017 at the commercial sheep farm “Poza Rica”, which is located in a temperate area named Singuilucan, in central Mexico. A total of 60 multiparous healthy and in good body condition (3.0) ewes were used, from which 30 were Suffolk x Hampshire (T1; wool type) and the other 30 were Katahdin x Pelibuey (T2; hair type). The ewes were synchronized with intravaginal sponges containing 20 mg of micronized cronolone (Chrono Gest, Intervet, Netherlands), which were inserted for 12 days. On day 10, the ewes received intramuscularly 400 IU eCG (Novormon, Sanfer, Mexico). The estruses were detected every 6 h with two fertile Katahdin rams equipped with an apron, starting 18 h after sponge removal. The time of estrus was recorded. On day 6 after estrus detection, just before embryo transfer, ovulation rate was determined as the number of corpora lutea observed in ovaries during laparoscopy. The recipients received two embryos of transferable quality (compact morula or blastocyst) within 3 h after its collection, coming from Charollais donor ewes using a laparoscope and standardized procedures. The embryos recovered into holding medium (Syngro, Vetoquinol, Canada) were transferred using a Tom catheter in the ipsilateral horn to the ovary in which ovulation was recorded, and the presence of the best quality corpus luteum was determined based on its size. On day 60, pregnancy diagnosis was performed using an ultrasound and a 3.5 MHz transabdominal probe (Aloka Prosound 2, Japan). The results of the incidence of estrus and pregnancy rate were analyzed as categorical variables with the CATMOD procedure, and ovulation rate with the ANOVA procedure, both of them available in SAS. The total percentage of estrus was similar ($p>0.05$) between ewes of T1 and T2 (95 and 100%). The incidence of estrus was also similar ($p>0.05$) for ewes from T1 and T2 at 24 h (75 and 85%) and 30 h (25 and 15%). Ovulation rate for ewes of T2 was higher ($p<0.05$) than for ewes of T1 (2.26 ± 0.21 vs 1.80 ± 0.15). Also, pregnancy rate was lower ($p<0.10$) for ewes of T1 than for ewes of T2 (60 vs 80%). In conclusion, under the conditions of the study, the results showed the superiority of using hair type multiparous ewes as embryo recipients.

A081E OPU/IVF/ET

Prediction of pregnancy after transfer of bovine *in vitro* produced embryos based on recipients' blood plasma metabolomics

Pascal Salvetti[1], Julie Gatien[1], Susana Carrocera[2], David Martín-González[2], Marta Muñoz[2], Enrique Gómez[2]

[1]ALLICE, Nouzilly, France; [2]SERIDA, Gijón, Spain.

Keywords*:* cattle, embryo recipients, metabolomics.

In association with genomic selection schemes, embryo transfer (ET) of *in vitro* produced embryos (IVP) is steadily increasing worldwide in cattle, although calving rates remain lower than from *in vivo* embryos. Thus, identification of competent embryos and recipients able to reach pregnancy at term is a major objective in reproductive biotechnology. However, practitioners lack indicators to select suitable recipients, often leading to the exclusion of fertile animals. In that context, this study aimed to identify metabolite biomarkers in blood plasma of recipients belonging to several breeds (dairy, beef and crossbreed), that could predict pregnancy after ET of fresh or vitrified IVP embryos. Blood plasma of 130 recipients (67 Holstein for the training dataset; 63 for the validation dataset including 17 Holstein, 21 Asturiana de la Montaña and 25 crossbred) was collected at Day 0 (estrus) and Day 7 (4 to 6 hours prior to ET) and stored at -150° C until nuclear magnetic resonance (NMR) analysis. On Day 7, fresh (N=67; 34 for training and 33 for validation) or vitrified/warmed (N=63; 33 for training and 30 for validation) IVP embryos were transferred and pregnancy status was evaluated by trans-rectal ultrasound scanning at Day 40, 62 and at birth. NMR analysis led to absolute quantification of 36 metabolites. Average pregnancy rates were respectively 53.8 (50.7 for fresh and 57.1 for vitrified), 49.2 (44.8 for fresh and 54.0 for vitrified) and 43.8% (40.3 for fresh and 47.6 for vitrified) at Day 40, 62 and birth with no statistical differences between fresh and vitrified embryos. Data were examined for normality with Univariate procedure (SAS/STAT software). Thereafter, metabolites differentially expressed between pregnant and open recipients were identified by General Linear Model for each metabolite and each pregnancy checkpoint. Differences were considered significant at $p<0.05$ and $FDR<0.05$. Interestingly, putative biomarkers were only identified on Day 7 or by subtracting Day 0 and Day 7 (only for vitrified embryos) but not at Day 0. Biomarkers for fresh embryos were consistently identified on Day 40, Day 62 and birth, while vitrification led to a marked drop in biomarker abundance at birth. Overall classification accuracy was calculated to identify three types of biomarkers: 1) independent of breed and embryo type (2-Oxoglutaric acid; Ornithine); 2) specific for fresh embryos (L-Alanine, Ketoleucine, L-Threonine, 3-methyl-2-oxovalerate, Propionic acid); and 3) specific for vitrified embryos (L-Glycine, L-Glutamine, L-Methionine, L-Lysine). Metabolic enrichment analysis distinguished between recipients for fresh (enriched energy oxidative metabolism from fat in pregnant recipients) and vitrified embryos (low enrichment in lipid metabolism in pregnant recipients). Recipient selection by their pregnancy probability in a defined cycle seems to be possible using the biomarkers here identified for the first time. These findings may allow reliable recipient selection according to the cryopreservation status of the embryo, thus optimizing the efficiency of breeding programs.

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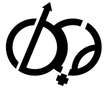

A082E OPU/IVF/ET

Short term temperature elevation during IVM affects embryo yield and alters gene expression pattern in oocytes, cumulus cells and blastocysts in cattle.

Constantina Stamperna[1], Eleni Dovolou[1], Themistoklis Giannoulis[1,2], Zissis Mamuris[2], Katerina Dadouli[1,3], Georgios S. Amiridis[1]

[1]Univeristy of Thessaly, Veterinary Faculty, Karditsa, Greece; [2]University of Thessaly, Faculty of Biochemistry and Biotechnology, Larissa, Greece; [3]University of Thessaly, Faculty of Medicine, Larissa, Greece.

Keywords: heat stress, embryo, cattle.

Heat stress causes subfertility in cattle by inducing endocrine disruptions and deteriorating of oocyte and embryo quality. In this study we evaluated the effects of short lasting, moderate temperature elevation during IVM, on embryo yield, and on the expression of various genes. Abattoir derived oocytes were matured for 24 hours in TCM199 plus FCS and EGF at 39°C (controls n=549) or at 41°C from hour 2 to hour 8 of IVM (treated, n =867). Matured oocytes were fertilized by frozen/thawed swim-up separated sperm. Presumptive zygotes were denuded and cultured at 39°C in SOF supplemented with FCS for 9 days in microdroplets in groups of 25. In 8 replicates, cleavage and blastocyst formation rates were evaluated at 48 hours PI and on days 7,8,9 respectively. Cumulus cells, oocytes and blastocysts from 5 replicates were snap frozen for gene expression. qRT-PCR was used for analysis of expression pattern of genes related to metabolism, thermal and oxidative stress response, apoptosis, and placentation in oocytes (7 genes), cumulus cells (12 genes) and blastocysts (11genes). Three reference genes (YWHAZ, EEF1A1, UBA52) were used to normalize gene expression values per sample using their geometric mean and their suitability for normalization was checked with the geNorm program. Cleavage, embryo formation rates and gene expression between treated and control groups were tested by 2tailed students t-test. Correlation analysis was performed by bi-clustering the samples according to their origin and their condition, which is an appropriate method for functionally heterogeneous data. Correlation and regression analysis were performed using gene expression data between groups, by the functions cor and rcorr implemented in R.

In treated group, cleavage and embryo formation rates were lower compared to controls (cleavage 86.7% vs 74.2%; blastocysts: day 7, 29.9% vs 19.7%, day 8, 34.2% vs 22.9% and day 9 35.9% vs 24.5%), in all cases $p<0.001$. Relative mRNA abundance of *HSPA1A, HSPB11, HSP90AA1, GPX1, GLUT1, PTGS2, GREM1, CPT1, G6PD, LDHA, CCNB1, MnSOD* in cumulus cells, *HSPA1A, HSPB11, HSP90AA1, G6PD, GPX1, CCNB1, MnSOD* in oocytes and *HSPA1A, HSP90AA1, DNMT3A, PTGS2, ACR1B1, PLAC8, GPX1, MnSOD, GLUT1, IGF2R, BAX* in day 7 blastocyst was measured by RT-PCR. No statistically significant difference was detected in any gene between treated and control groups. Heat treatment affected ($p<0.05$) the correlation of expression between *HSPB11 and G6PD, GPX1* and CCNB1 in oocytes. In cumulus cells HSP90AA1 was negatively correlated with *HSPA1A, LDHA* and *CCNB1,* while *CCNB1* was positively corelated with *HSPA1A, LDHA, GPX1* and *G6PD*. In blastocysts, heat treatment caused a negative correlation between *HSP90AA1, AKR1B1* and *PLAC8*. These results imply that exposure of oocytes to elevated temperature even for only 6 hours dramatically reduces the developmental competence of the oocytes, suppresses blastocyst yield and disrupts the coordinated pattern of a series of gene expression.

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A083E OPU/IVF/ET

Effect of antioxidant α-tocopherol on bovine oocyte's maturation

Loide Valadão, Maria Moreira da Silva, Fernando Moreira da Silva

University of the Azores, Faculty of Agrarian Sciences and Environment - IITAA - Portugal.

Keywords*:* cattle, oocyte maturation, alpha-tocopherol.

Vitamin E is an important natural antioxidant, and its most common and biologically active form is the α-tocopherol, being well known as a scavenger of free radicals in a hydrophobic milieu. As the chain-breaking of this antioxidant has not been reported to be present in mammalian spermatozoa, the present work was designed to evaluate the effect of the α-tocopherol on the maturation rate of bovine oocytes. For this purpose, 194 bovine ovaries divided by 12 replications, were collected at the slaughterhouse in the Terceira Island and transported to the laboratory. Follicles 2 to 8 mm in diameter were punctured, 779 cumulus-oocyte complexes (COCs) considered of quality 1 and 2 according to their morphological aspect, were assigned to maturation and randomly divided into 4 groups. Each oocyte group was maturated in a standard TCM 199 medium supplemented with fetal bovine serum, FSH/LH, Estradiol, Glutamine and Sodium pyruvate, added with different concentrations (0, 0.5, 1 and 2 mM) of α-tocopherol for 24 h at 38.5 °C, with 5% CO_2 in the air and saturated humidity. Then, COC's, cumulus cells were removed, the oocytes were stained with aceto-orcein, observed under the phase contrast microscope, and the different nuclear phases were evaluated from profase I to Metaphase II. *In vitro* maturation results are expressed as a percentage of oocytes reached the Metaphase II stage. Statistical differences among treatments were evaluated by the ANOVA test. α-Tocopherol at a concentration of 0.5mM increased significantly ($P<0.05$) the maturation rate, relative to the control group, 68.0% vs 60.8%, respectively. At a concentration of 1 and 2 mM, no significant differences were observed when compared to the results obtained in the control group, with maturation rates of 64.3 % and 60.6%, respectively. This study clearly suggests addition of **α**-tocopherol at a concentration of 0.5 mM increases the maturation rate of bovine oocytes, despite it did not reveal the mechanism by which the antioxidant acts to improve maturation results. Further studies on possible effects of different concentrations of this antioxidant on the developmental competence of *in vitro* produced embryos (IVM/IVF), and the viability of these embryos after transfer to recipient heifers on Day 7 post-estrus will be evaluated in our future research. Studies on vitamin E supplementation of bovine females will be also implemented.

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A084E OPU/IVF/ET

Influence of selected factors on the effectiveness of the embryo transfer in cows

Maria Wieczorkiewicz[1], Magdalena Herudzińska[1], Jarosław Czeladko[1], Jakub Kulus[1], Ewa Wędrowska[3], Bartłomiej Maria Jaśkowski[2], Magdalena Kulus[1], Marek Gehrke[1], Jędrzej Maria Jaśkowski[1]

[1]Center for Veterinary Sciences, Nicolaus Copernicus University in Torun, Torun, Poland, Poland; [2]Department of Reproduction and Clinic of Farm Animals of the Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Wroclaw, Poland; [3]Department of Applied Informatics and Mathematics in Economics, Faculty of Economic Sciences and Management, Nicolaus Copernicus University in Torun, Torun, Poland.

Keywords*:* embryo transfer, recipients, risk factors.

The objective of the study was to identify risk factors associated with CR(conception rate) after ET(embryo transfer) in cows. It involved 952 cases of ET. Embryos were obtained from previously selected, hormonally synchronized (200 µg of cloprostenol) and superovulated (30 mg of pFSH in 8 decreasing intramuscular doses within 4 days and then 2 intramuscular injections of 500 µg cloporstenol) donors using a bloodless method (embryo flushing). Embryos' selection was carried out according to the evaluation of their morphology. Only embryos in stages of development 3-6 and quality 1-3 were used in the study (Bo 2013). Excellent quality embryos in the morula stage (n= 667) were intended for the direct transfer. Other embryos (n= 285) were destined for freezing and prepared for direct transfer after thawing. The group of embryo recipients consisted of 952 heifers of the Polish Holstein-Friesian breed with mean age 15.5 months and mean body condition score (BCS) 3.02. The heat was synchronized using 2 intramuscular injections of 500 µg cloprostenol given at 14-day intervals. Based on the ultrasound examination females having corpora lutea with diameter above 15 mm qualified for the ET. Embryos were placed in the recipients' uteruses between 6 and 8 days after synchronized or natural heat (757 vs. 195 heifers). The transfer procedure was performed with the use of Wörrlein gun (Goldenpick type) placed in the plastic sanitary case (Minitüb). Embryos were always inserted in the uterine horn ipsilateral to the ovary with the CL. After 2 months all recipients were clinically examined (transrectal palpation) for the pregnancy. It investigated the effect of season on ET, embryo quality or type (fresh vs. frozen), recipient's age and BCS, day of embryo introduction, depth of embryo insertion in the uterine horn, duration of the gun passage through the cervix, horn of the uterus (left vs. right), size and type of the corpus luteum (solid vs. cavernous) and type of treatment used before ET (hCG, flunixin meglumine (FM) and a combination of FM and hCG).To identify the determining factors of CR, the multivarious logit model was estimated using STATA software. The pregnancy was confirmed in 419 after fresh and 159 after frozen embryo transfer cases out of 952 of recipients (CR respectively: 62.8 and 55.8; total CR= 60.7%). The season as well as the embryo development stage were distinguished as statistically significant ($p < 0.05$). The best results (CR= 70%) were obtained in spring, the weakest in summer (CR= 50.2%). Most embryos were transferred at the stages "5" and "6" (n= 451, CR= 62.9). The more developed the transferred embryo was, the higher the CR in recipients. While embryos at the stage "4" led to pregnancy of 53% of the recipients (n=114), embryos at the stage of development "7" and "8" resulted in 66.7% and 83.3% of pregnancies respectively. However, embryos in these stages amounted only to 1.89% (n= 13). The significant ($p < 0.01$) impact of the 'condition' variable was also observed. The highest CR (67.6%) was noticed in recipients with 3.5 points (n= 157) and the lowest - 45.2% - in recipients with BCS higher than 4 (n= 42). The influence of other analyzed variables was not statistically significant.