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Possibilities of preserving the local cow breeds in Latvia

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Keywords: cow endometrium, reproduction, embryo transfer.

Latvian Brown (LB) and Latvian Blue (LZ) are local Latvian breeds of the bovine gene-fund (GF) and therefore unique and characteristic only to Latvia. In 2016, only 152 LB and 313 LZ cows were registered as GF animals. So far, natural breeding and artificial insemination (AI) have been the methods of conserving GF in Latvia. Majority of the GF animals are at advanced age, they live on small farms without calculated feeding ration, and therefore may suffer from reproductive disorders. In 2017, through ERAF project Nr. 1.1.1.1/16/A/025 „Latvian Brown and Latvian Blue cow gene pool conservation using embryo transfer (ET) and related biotechnologies", ET will be restarted in Latvia after 35 years of interruption. The aim of this investigation was to consider possibilities of using ET in LB and LZ cows that could be classified as inferior embryo donors. The analysed data was obtained in Latvia in 1984-85. Thirty Holstein-Friesian cows of which 22 (73.3%) were 9-10 years old were removed from their herds due to reproductive problems (83%) or mastitis (17%) and used as embryo donors. Uterine biopsies were taken from 30 cows on Days 8-9 i.e.1-3 days before superovulatory treatment initiated. Results are presented as average ± standard error and Student's t-test was used to compare two independent samples. Histological investigation revealed different types of endometrial alterations in 91.7% of the cows. A different degree of endometrial oedema and hyperaemia were observed in 23 (76.7%) cows. Despite the fact that vascular fibrosis, hyalinosis concurrently with lymphoid-histiocytic infiltration in stratum compactum of the endometrium were established in 12 (40%) cows, superovulation was reached in 9 (75%) and the amount of corpora lutea (CL) was 6.3±1.42. Embryos of excellent or good quality (0.9±0.45) were obtained from 5 (41.7%) cows. Eighteen cows without strongly expressed endometrial pathologies had successful superovulatory response (CL 8.6±0.82) with 4.5±0.96 embryos of which 3.5 ± 0.66 were viable. In cows with or without serious endometrial pathologies, the number of CL was equal ($P > 0.05$), but the number of good quality embryos differed significantly ($P < 0.05$). Also, in recipients receiving embryos from relatively healthy cows versus embryos from cows suffering from serious endometrial pathologies, the viability of transferred embryos significantly differed ($P < 0.05$). In general, a successful superovulatory response occurred in 22/30 (73.3%) cows. No embryos were obtained from 9 (30.0%) cows. After 2 months, pregnancy was approved in 49% of recipient heifers. Lymphoid-histiocytic, plasmatic and mast cells infiltration in endometrial subepithelium and stroma as well as vascular hyalinosis and fibrosis had a significantly negative influence on embryo recovery. It is profitable to conduct a histological investigation of uterine tissue alongside with other analyses if a cow is considered as an inferior embryo donor due to its health and general condition.

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Artificial activation of ovine oocytes is required after ICSI with freeze-dried spermatozoa

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Keywords: freeze-drying, ICSI, spermatozoa.

Freeze-drying allows to store the biological samples in a dry state and represents an interesting alternative low-cost strategy of semen biobanking to save the endangered species. Here, we have established a dry sperm biobank from an endangered Italian sheep breed (Pagliarola) and tested its efficiency through ICSI. The motile spermatozoa from ram ejaculates collected with artificial vagina was selected by swim-up in TRIS-based medium (2.42g TRIS, 1.36g citric acid, 1.00g fructose, 100.000 U.I. penicillin G, 100mg streptomycin, in 67.20ml bidistilled water (ddH_2O); pH was adjusted to 6.7) for 20 minutes at 38.5°C. The motile spermatozoa were frozen in freeze-drying medium (10mM EGTA and 50mM NaCl in 10mM Tris–HCl buffer; pH was adjusted to 8.4) in a -80°C freezer for 75 minutes and subsequently lyophilized by the freeze-drying apparatus SP Scientific-VirTis, Freeze-dryer 2.0 BenchTop, 20 hours with a condenser temperature of -58°C and vacuum of 20 mTorr). The vials were sealed in glass vials under vacuum and stored in the dark at 4°C for 1-2 months. Just before the ICSI, the freeze-dried spermatozoa were rehydrated by adding 100µl ddH_2O. To evaluate the fertilizing capability of freeze-dried spermatozoa, 108 MII sheep oocytes were subjected to ICSI and allocated to two groups: 56 oocytes were activated by incubation with 5µM ionomycin (ICSI-FDS*a*); 52 were left un-activated (ICSI-FDS*na*). Forty-four oocytes injected with frozen spermatozoa (ICSI-FS) and left un-activated, served as control. Pronuclear formation (2PN) and blastocyst development were investigated at 14-16 hours and 7-8 days after ICSI, respectively. Differences were considered statistically significant for $p<0.05$ (*Chi-square* test). Data were analyzed using PRISM, software version 5.0; GraphPad. The freeze-dried spermatozoa were completely immotile after rehydration, however they maintained the capacity to fecund oocytes after ICSI. Two PN were found in 83.3% of ICSI-FDS*a,* 81.4% of ICSI-FS while only in 14.3% of ICSI-FDS*na* ($p<0.05$ ICSI-FDS*na* vs ICSI-FDS*a; *$p<0.01$ ICSI-FDS*na* vs ICSI-FS). Likewise, the ICSI freeze-dried spermatozoa yielded blastocysts only following artificial activation (ICSI-FDS*a*: 10.2%; ICSI-FS: 31%; ICSI-FDS*na*: 0%; $p<0.05$ ICSI-FDS*a* vs ICSI-FDS*na* and ICSI-FS; $p<0.0001$ ICSI-FDS*na* vs ICSI-FS). Our finding show that freeze-dried spermatozoa have lost the capacity to trigger oocyte activation but maintained their nuclear viability, whose developmental potential was fully released following artificial activation. Our results support the evidence that freeze-drying effective approach of spermatozoa storage to save endangered species. This work was supported by DRYNET H2020-MSCA-RISE-2016 and ERAOFART-H2020 -TWINN-2015.
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AVEN and BCL-xL expression pattern and protein-protein interaction assessment through bovine early embryo development

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Keywords: oocyte, embryo, apoptosis.

Apoptosis in embryonic cells is important for embryo development; stabilizing cell numbers and playing a role in cell quality control. However, it is also associated with embryonic loss and cellular response to suboptimal developmental conditions and stress. AVEN, a novel P4-regulated protein, inhibits the mitochondrial apoptosis pathway by binding to and enhancing antiapoptotic BCL-xL activity. The objective of this study was evaluate the protein expression profile and protein-protein interaction of AVEN and BCL-xL during early embryo development in cattle. Briefly, cumulus oocyte complexes were recovered from abattoir derived ovaries and submitted to in vitro embryo production (IVP). Six replicate sets of samples were retrieved from IVP at 7 different developmental stages (Germinal vesicle (GV), Metaphase II (MII), zygote (16 h), 2-cell (44 h), 8-cell (72 h), compact morula (Day 5) and blastocyst (Day 7), -post insemination (pi)), and fixed in 4% paraformaldehyde. Samples were then processed for Proximity Ligation Assay (PLA) or whole-mount fluorescent immunocytochemistry: PLA detection of Aven-BCL-xL interaction was achieved using the DUOLink™ In Situ Red Starter Kit reagents according to the manufacturers (Sigma-Aldrich) instructions. Whole-mount immunofluorescence: Following washing and blocking, samples were incubated overnight with a single: monoclonal mouse (m) anti-AVEN (1:400, Abcam, ab77014), or polyclonal rabbit (r) anti-BCL-xL (1:400, Abcam, ab2568); dual: (mAven & rBCL-xL) and negative control: none, primary antibodies. Secondary antibodies Alexafluors- 594 goat anti-mouse and 488 goat anti rabbit were employed at a dilution of 1:400, for immunofluorescent labelling. Fluorescent labelling was observed under epifluorescent and confocal laser scanning -microscopy. The number of PLA Aven-BCL-xL interaction foci was counted per unit area of oocyte or embryo sample at each developmental stage (n= >14, per stage), using ImageJ software. Data was analysed using a One-Way ANOVA followed by post-hoc Tukey's test. There were significantly more foci in blastocysts compared to GV oocytes, 2-cell, 8-cell and morula -stage embryos ($P<0.05$, mean and SEM = 332,8 ± 56,04 vs. 158 ± 27,94, 162,7 ± 16,98, 160,1 ± 14,02 and 80,34 ± 13,67, respectively). The lowest number of interaction foci was detected in compact morulas (80,34 ± 13,67), this was significantly lower than MII oocytes (213 ± 22,12), zygotes (273,5 ± 41,68) and Blastocysts (332,8 ± 56,04). A distinct labelling pattern was observed during development; foci were evenly distributed in the cytoplasm of GV and MII oocytes, zygotes, 2-cell and 8-cell -embryos, but restricted mainly to peripheral cells in compact morulas and to the trophoblast cells of blastocysts. These results were corroborated by the co-localization pattern of AVEN and BCL-xL in the whole-mount samples. This is the first study to employ PLA for the analysis of protein to protein interaction in bovine oocytes and embryos and shows a very precisely regulated interaction of AVEN and BCL-xL during bovine embryo development.

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Caspases and TNFa activation in sperm storage tubules is correlated with hen's fertility

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Keywords: sperm storage tubes, Caspase, TNFa.

Blebbing is a basic event of apoptotic cell death mediated by caspase proteins. Used by cells to package cytoplasm portions, organelles or DNA, this biochemical cascade can also be used by secreting cells to release vesicles by Actin–Myosin II contraction (Mills et al., J. Cell Biol., 146(4):703–707, 1999). Blebbing does not occur in some cells lacking caspase 3 (Janicke et al., J. Biol Chem. 273:9357–9360, 1998; Zheng et al., Proc. Natl. Acad. Sci. USA. 95:13618–13623, 1998). Avian sperm storage tubules (SST) are apocrinal tubular glands located in the hen's utero-vaginal junction (UVJ) that store spermatozoa for long period (Fujii and Tamura, J. Fac. Fish. Anim. Husb. 5(1): 145-163, 1963). In this work, we investigated the correlation between capases activation in SST cells and hen's fertility. Hens from two divergent fertility lineages with high (DF+=21 days) and low (DF-=10 days) period of sperm storage (Beaumont C., J. An. Sci. 72:193-201, 1992.) were artificially inseminated (AI) (200 x 10^6 sperm). Animals (3 hens per condition) were sacrificed without insemination (control), and at 24 hours, 1 wk, 2 wks and 3 wks after AI. Paraffined UVJ tissue was prepared for immunohistochemistry (IHC) against Tumor Necrosis Factor a (TNFa), Caspase-3 (CASP-3), and -8 (CASP-8). The same antibodies were used for Western blotting (WB) quantitative analysis. Statistical analysis was performed by Kruskal-Wallis test and Turkey post-test, the significance threshold was set at $p<0.001$. Protein quantitative analysis (WB) reveals that CASP-3 was 1.3-fold lower in control DF+ than in DF-. At 24 h after AI, CASP-3 was 1.7-fold higher in DF+ than in DF-. CASP-8 was 6.4-fold higher in control DF+ than in DF-, 5.3- and 10.4-fold lower after 1 wk and 2 wks of insemination, respectively, and 1.6-fold higher after 3 wks of insemination. TNFa was 3.4-fold lower in control DF+ than DF-, similarly to caspases, and 2.5- and 1.3- and 3.6- fold higher after 24 hours, 2 wks and 3 wks of insemination, respectively. TNFa, CASP-3, and -8 were observed (IHC) inside UVJ in all moments in both lineages. CASP-3 and CASP-8 were detected with high intensity in external UVJ epithelial surface, and in SST cells of control DF+ and DF- hens. After 24 hours of insemination, CASP-3 and CASP-8 were observed specifically in DF- SSTs and DPF+ SSTs, respectively. TNFa was strongly distributed in connective tissue in association with SST cells as well as in surface epithelia at all moments. These results suggest that caspase activation in SST cells can be correlated with hen's fertility. Rapid caspase activation (within 24 hours) correlated to TNF pathway after insemination could allow longer period of sperm storage.
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Effect of non-esterified fatty acids during *in vitro* oocyte maturation on the development of bovine embryos after transfer

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Keywords: NEFA, oocyte, embryo transfer.

Metabolic disorders, as in negative energy balance (NEB) dairy cows, are associated with elevated non-esterified fatty acid (NEFA) concentrations, predominantly palmitic acid (PA), in the follicular fluid. These NEFAs are known to jeopardize oocyte *in vitro* maturation and elicit altered blastocyst quality and physiology. Lipotoxic conditions during final oocyte maturation also influence epigenetic reprogramming in the resultant day (D) 7 embryo and may thus affect subsequent development, potentially imprinting lasting marks during later stages of life. Therefore, we hypothesized that exposure of oocytes to high NEFA concentrations during IVM affects post-hatching development of D7 blastocysts after embryo transfer. Bovine oocytes were matured for 24h under 2 conditions: 1) physiological NEFA conditions (28μM stearic acid (SA), 21μM oleic acid (OA), 23μM PA (BAS) and 2) elevated PA concentration as present in follicular fluid during NEB (150μM) with physiological concentrations of SA (28μM) and OA (21μM) (HPA). Matured oocytes were routinely fertilized and cultured in SOF with serum until D7. Cleavage (D2) and blastocyst rate (D7) were compared among treatments using a binary logistic regression model. Eight blastocysts (normal and expanded, equally distributed per treatment and per replicate) were transferred per cow (n=8, 5 replicates). Four cows were attributed to HPA or BAS per replicate and were crossed over for the next replicate. Embryos were recovered at D14 and morphologically assessed (n=46). Glucose, lactate and pyruvate turn-over and interferon-tau (IFNT) secretion were measured in extra-embryonic tissue (EXT) after 24h culture (n=62). Morphological, metabolic and IFNT data were tested for normality with a Kolmogorov-Smirnov test and differences between treatment were analysed with a T-test. Data are presented as mean ± SEM. Developmental competence at D7 was not significantly different between treatments (blastocyst rate of 26 vs. 29.6% for HPA and BAS, resp.). Recovery rate at D14 was 30% and 36% for HPA and BAS, resp. (P>0.05). HPA during IVM significantly reduced embryo elongation (3.7±1.5 vs. 8.6±1.7mm, P=0.001) but did not affect diameter of embryonic disc compared to BAS. EXT from HPA group consumed similar amount of glucose but tended to produce less lactate compared to EXT from BAS group (1732±211 vs. 2428±355pmol/mm^2/h, P=0.073). IFNT secretion was significantly lower in HPA group (0.47±0.71pg/ml) compared to BAS group (3.79±1.16pg/ml, P=0.018). In conclusion, exposure to elevated PA during *in vitro* oocyte maturation affected post-hatching development at D14. Embryos were less elongated, were metabolically altered and produced less IFNT, a major signal of pregnancy recognition, than their physiological counterparts. This suggests that metabolic stress during oocyte maturation may have long-lasting effects on embryo development that may lead to higher pregnancy loss and reduced fertility in high yielding dairy cows. More research is ongoing to investigate underlying mechanisms through genome wide transcriptome pathway mapping.

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Hypothermic storage (4?C) of ovine embryos with different medium and duration

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Keywords: ovine, embryo, hypothermic storage.

Generally, sheep embryos are transferred immediately after collection, however developing of a culture media for successful cooling and short-term storage would have enormous theoretical and practical implications in sheep embryo transfer programs (Romao et al., 2016). Differences in species' cryosensitivity of embryos are responsible for different approaches in their cryopreservation. It was reported that, one of the major concerns in embryo cryopreservation is its lipid content that can hamper the process (Dattena et al., 2000), as it happens in species such as sheep or pigs. Embryo lipid content effect on chilling sensitivity is not totally elucidated at the moment. In cattle, Ideta et al., (2013) introduced a medium that bovine embryos can be held for up to 7 days at 4°C and pregnancy rate of 75% was obtained for embryos held in this medium and transferred to primed recipients. The objective of this study was to determine if the commercial holding media (SYNGRO, S) and hypothermic medium (199 plus 50% FBS supplemented with 25 mM HEPES) could maintain viability of ovine embryos during extended hypothermic storage at 4°C in vitro as assessed by transfer to synchronized recipients. Donor ewes from prolific breeds (n=26) were superovulated to recover embryos on day 6 and embryos with grade 1 to 2, morula and blastocyst-stage embryos were sorted in holding media (SYNGRO, S) at room temperature (22°C). The embryos were then allocated at random to different treatment groups within 2 h of collection. T1 embryos (n=80) were washed three times in hypothermic medium (199 plus 50% FBS supplemented with 25 mM HEPES) and loaded into a plastic straw (1/4 cm3 clear straw; 4-6 embryos/straw). T2 embryos (n=20) were stored in commercial holding media (SYNGRO, S) in a plastic straw (1/4 cm3 clear straw; 4-6 embryos/straw). Then a water jacket was prepared by placing a number of 10mm goblets into a 65mm goblet filled with room temperature tap water and with wet cotton or gauze to keep the small goblets from floating/tipping. The holding container was placed in a 4°C fridge and kept adding straws of embryos for at least 24h. Then, 65mm goblet bath was be placed in a Styrofoam box with ice packs for air transportation. Upon the arrival, embryos were placed in a 4°C fridge for 168h in T1 group and 48h in T2 group. For transfer, the straws were kept at 4°C until needed and were them emptied into a dish of fresh holding media at room temp and then loaded into IVF catheters as they are transferred twin into the recipients (number of recipients for T1 embryos=40; number of recipients for T2 embryos=10). Following hypothermic storage for 7 d (T1) and 2 d (T2), embryos were transferred into recipients by laparoscopy to the uterine horn ipsilateral to a corpus luteum on day 6, and survival was determined on day 50 by ultrasound. There was no pregnancy established with either T1 or T2 group of embryos. It was concluded that these two techniques were not found successful for enabling liquid nitrogen-free storage and air transportation of embryos.

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Bovine endometrial cells are responsive to embryonic sex *in vitro*

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Keywords: embryo, sex, endometrial response, *in vitro* culture.

Bovine endometrium recognizes embryonic sex at early stages with multiple embryos in vivo (Gómez et al, J Proteome Res 12:1199-210). In this work, we investigated the influence of embryonic sex on carbohydrate, protein and gene expression of endometrial cells co-cultured in vitro (EC) with a single embryo. Day-6 in vitro-produced morulae were individually cultured for 48h on the epithelial side of ECs. EC consisted of epithelial cells grown in inserts plated on stromal cell cultured in wells with TCM-199. Day-8 expanded blastocysts were collected and sexed by amelogenin gene amplification. Samples of EC and conditioned media were cultured with one male embryo (ME), one female embryo (FE) and no embryo (negative control; C). Samples were collected from 4 uterus as follows: uterus-1, 2 ME, 1 FE, 1 C; uterus-2, 2ME, 1 FE, 1 C; Uterus-3, 2 ME, 0 FE, 1 C; Uterus-4, 0 ME, 2 FE, 1 C. Expression of genes (N=13) coding for growth factors, receptors for hormones that regulate estrus cycle, receptors that bind embryonic signals, and metabolism, were analyzed in epithelial and stromal cells. Concentrations of glucose, fructose, lactate, artemin protein and total protein were determined in conditioned medium from the epithelial side. Data were analyzed using GLM and REGWQ Test and Principal Component Analysis (PCA). The relative mRNA abundances for candidate genes were compared using ANOVA y All Pairwise Multiple Comparison (Student-Newman-Keuls Method). Embryos altered transcription only in epithelial cells, not in stromal ones. Thus, expressions induced by ME were lower ($P<0.01$) than FE and controls (C) in hexose transporters solute carrier family 2 member 1 (*SLC2A1*: M=1.00±0.12, FE=6.05±0.07, C=7.19±0.10) and member 5 (*SLC2A5*: M=1.00±0.13, FE=5.58±0.08, C=7.17±0.11), connective tissue growth factor (*CTGF*: ME=1.00±0.22, FE=3.13±0.15, C=2.85±0.09), interferon alpha and beta receptors subunit (*IFNAR1*: ME=1.00±0.18, FE=2.46±0.29, C=2.86±0.15; *IFNAR2*: ME=1.00±0.17, FE=2.30±0.10, C=2.24±0.29). Male embryos elicited lower expression of artemin (*ARTN*) than FE (ME=1.00±0.26, FE=4.53±0.15, $P<0.05$) and controls (ME=1.00±0.26, C=3.74±0.30; $P<0.01$). Female embryos reduced ($P<0.01$) *SLC2A1* and *SLC2A5*, and increased ($P<0.05$) *ARTN* expression with respect to controls (*SLC2A1:* FE=6.05±0.07, C=7.19±0.10; *SLC2A5:* FE=5.58±0.08, C=7.17±0.11; *ARTN*: FE=4.53±0.15, C=3.74±0.30). ARTN protein and gene expressions strongly correlated ($R>0.90$; $P<0.05$) in the group of ME or FE, but not in controls. Embryonic sex did not alter hexoses or lactate concentrations in EC-conditioned medium. The concentrations of carbohydrates and expressions of genes that showed sexual dimorphism covariated significantly (|0.429971|; PCA). In contrast total protein mainly covariated with expressions of estrogen and progesterone receptors. Isolated male and female embryos may differentially release signaling factors that induce sexually dimorphic responses in endometrial cells. 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).

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Transcriptomic response of bovine oviduct epithelial cells to the early embryo

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Keywords: BOEC, transcriptome, embryo.

Previous data from our group and others indicate that the early bovine embryo may be more than just a passive structure during its journey through the oviduct, and may in fact elicit a transcriptomic response from the oviduct epithelial cells. However, such an effect is likely to be very local in nature, making it challenging to detect *in vivo*. In order to examine the possible embryo effect on BOEC transcriptome and whether it is local or not, we used an *in vitro* model involving co-culture of early embryos with a monolayer of bovine oviduct epithelial cells (BOEC). Oviducts corresponding to the early luteal phase were collected from the slaughterhouse and BOEC were mechanically harvested from the isthmus and cultured in 500 µl of Tissue Culture Medium-199 supplemented with 10% fetal calf serum (FCS) in four-well culture plates in a humidified atmosphere at 5% CO_2 in air at 38.5°C during 6 days until confluence. A day before co-culture the medium was replaced with synthetic oviduct fluid (SOF) supplemented with 10% FCS. Embryos (n=50) at the 2- to 4-cell stage (Experiment 1) or at the 8-cell stage (Experiment 2) were cultured on BOEC in a polyester mesh to maintain the position of the embryos on top of the cells. After 48 h of co-culture, the cells directly beneath the embryos and those in the same well but located away from the embryos (i.e., not in direct contact) were recovered as well as cells from a control well without embryos. BOEC were snap frozen from 5 replicates and they were analyzed by qPCR to assess the expression of 12 candidate genes. These included oviduct genes previously shown to be affected by the presence of an embryo *in vivo* (Maillo et al. Biol Reprod. 2015. 92: 144) and *in vitro* (Schmaltz-Panneau *et al.* Anim Reprod Sci. 2014. 149(3-4):103-106). Statistical differences were assessed by ANOVA. Regardless of being in direct contact or not with 2- to 4-cell embryos, BOEC displayed a decreased abundance of *ARG3;* a gene implicated in the regulation of intracellular calcium and cytoskeleton organization; compared to control cells ($P<0.05$). Co-culture with 8-cell embryos also lead to an increased abundance of *ARG3* besides to others genes involved in BMP signaling pathway (*SMAD6*, *TDGF1*) and a decreased abundance of oxidative stress gene (*GPX4*) in BOEC whatever they have been in direct or indirect contact with embryos. While *SOCS3* a gene related to the inflammatory response was decreased in cells in direct contact with the embryos compared to both other groups ($P<0.05$). In conclusion, under our experimental conditions, the transcriptomic response of BOEC is embryo–stage dependent. For one gene, *SOCS3*, expression was only altered in BOEC in direct contact with the embryo. This may be due to a non-diffusible embryo-secreted factor.
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Effect of nutritional level on the onset of puberty in the Sardi ewe lamb: relationships with FSH, GH and Leptin

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Keywords: ewe lamb, nutrition, puberty.

The aim of this study was to investigate the possibility of a compensatory growth following starving and re-feeding of ewe lambs, their puberty onset and the related endocrine events in Sardi sheep.Thirty lambs born in autumn were assigned to 3 groups (10 animals per group): HH fed a high-level diet, LL a low-level diet and LH a low-level diet during 2 months (period 1) followed with a high level diet during 4 months (period 2). The low-level diet consisted of straw (500g) and concentrate (200g) while in high-level diet the concentrate (1kg per animal per day) and straw were fed ad libitum. The assay started on July the 1st when the lambs were 7 months old and an average weight of 22 kg, and finished on December the 30th.The lambs were weighed at birth, at weaning and at 2 week intervals thereafter until the end of the experiment. Blood samples were taken three times per week for measuring progesterone, FSH, GH and leptin concentration. Plasma P4 level > 1 ng/ml is a reliable indicator for an active corpus luteum and is related to cyclic ovarian activity.The main results showed a significant difference between growth rates of lambs on low or high diet but the average daily gain remained low 87 ± 9 g/d. The mean live weight at the end of experiment of HH lambs was significantly higher compared to LL and LH lambs (37.4±0.5 vs 24.8±2.7 vs 33.3± 3.3 kg respectively, $p< 0.05$) with HH lambs being 12kg heavier than those of LL group. Similarly, LH lambs were significantly heavier than LL lambs (33.3± 3.3 vs 24.8±2.7 kg, $p<0.05$). There was a partial compensatory growth in fasted-reefed lambs.The onset of puberty was mainly related to the live-weight in all ewe lambs. Thus, the average live weight at puberty was 31.4 ±0.7 kg and 29.8 ± 1.2 kg in HH and LH groups, respectively. In the LL group, however, no ewe lamb reached the puberty at 400 days, but the average LW was then only 25 kg. Before first ovulation, an increase in FSH plasma level was observed (2.5±1.2 vs 0.20 ng/ml) in HH group and (3.4±2.2 vs 0.23 ng/ml) in LH group.The average plasma GH concentrations in the HH group seemed to be lower (20.7±1.03 ng/ml) than in the other groups (25.5±2.6 vs 33.4±3.6 ng/ml, respectively, in the LH and LL)./>No significant relationship was found between the age at puberty (320 ±18 vs 372 ± 9 days) and the plasma leptin levels (1.8±1.1 vs1.9±1.3 ng/ml) in the HH and LH groups, respectively. These results confirm the importance of body development on the appearance of puberty, and raise the possibility of a compensatory growth in the lambs. Elsewhere, FSH concentrations were not significantly altered by feed restriction. The observed absence of relationship between the age at puberty and the plasma leptin levels may reflect the presence of an other metabolite, rather than leptin, to signal the nutritional status to the reproductive axis in lamb.

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Elevated non-esterified fatty acid concentrations during bovine oviduct epithelial cell and zygote coculture hamper early embryo development

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Keywords: NEFAs, BOEC/zygote coculture, polarized cell culture system.

Maternal lipolytic disorders and the associated systemic rise of non-esterified fatty acids (NEFAs) have been suggested to affect oviduct physiology and functionality. An altered oviduct micro-environment may influence early embryo development, however its consequences remain largely unknown. Therefore, we hypothesize that elevated NEFAs in a polarized cell culture system hamper early embryo development. Furthermore, we state that effects will depend on the presence of bovine oviduct epithelial cells (BOECs) and the direction of NEFA exposure. In 4 repeats, early luteal BOECs were seeded at $1x10^6$ cells/mL in a polarized cell culture system. After reaching 100% confluency (day 7) monolayers were cocultured with 25 zygotes per insert in 100µL SOF with 10% FBS and 0.75% BSA for 96h. Hereto, bovine oocytes were matured and fertilized *in vitro* following standard procedures. During subsequent BOEC/zygote coculture in SOF, NEFA exposure (720µM containing 210µM oleic acid + 230µM palmitic acid + 280µM stearic acid) was implemented in 3 groups: 1) [APICAL NEFA] i.e. 720µM NEFA + 0.45% EtOH in the apical compartment, 2) [BASAL NEFA] i.e. 720µM NEFA + 0.45%EtOH in the basal compartment, 3) [A/B NEFA+] i.e. 720µM NEFA + 0.45%EtOH in both compartments. Treatments were compared to [SOLVENT+] i.e. 0.45% EtOH in both compartments with BOEC coculture, [A/B NEFA-] i.e. 720µM NEFA + 0.45%EtOH in both compartments but without BOEC coculture, and [SOLVENT-] i.e. 0.45%EtOH in both compartment without BOEC coculture. After 96h, all morulae were transferred to SOFmedium in a 96-well plate without BOEC. Embryo development was assessed using cleavage- (48h pi), morula- (120-126h pi), and blastocyst rates (192h pi). Data were analysed using binary logistic regression with Bonferroni correction in SPSS, and were considered statistically different when $P<0.05$. Total cleavage in A/B NEFA+ (51.63%) and A/B NEFA- (43.19%) differed significantly ($P=0.02$), and were lower compared to other treatments. From the cleaved oocytes APICAL NEFA showed an increased percentage of zygotes in 3-cell stage (17.61%; $P=0.032$). Morula rates were on average 28.05% out of total oocytes and 47% out of cleaved oocytes, and similar between all treatments ($P>0.05$). Blastocyst rates (out of total oocytes) were significantly higher in SOLVENT+ and SOLVENT- (26.11% and 22.67% resp) compared to NEFA treatments (12.59%; $P<0.001$). In all treatments, day 8 blastocysts were mostly in expanded stage (55.06%), except for APICAL NEFA which showed 48.14% young blastocysts. In conclusion, NEFAs negatively affect embryo developmental competence. During cleavage, but not at blastocyst level, these effects are limited to bidirectionally exposed groups, and the cocultivation with BOECs seemed to have beneficial effects. Data suggest that elevated NEFAs in the oviduct may attribute to the complex pathogenesis of sub- and infertility during lipolytic disorders, however, more research is required to further elaborate on potential compensatory effects mediated by the oviduct.

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Functional activity of actin cytoskeleton in porcine oocytes during *in vitro* maturation

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Keywords: oocyte, cytoskeleton, IVM.

Actin is an abundant protein with well established roles in fundamental processes ranging from cell migration to membrane transport (Sun Q. &Schatten H. Reprod. 131, 193–205, 2006). Brilliant cresyl blue (BCB) staining has been used for selection of the functional status of oocytes. BCB^+ oocytes (oocytes that have finished growth phase in vivo) had significantly higher development competence than BCB^- oocytes (oocytes that have not finished growth phase in vivo, Ishizaki C. et al., Theriogenology, 72(1): 72–80, 2009). The aim of the present study was to compare the functional activity of the actin cytoskeleton [the intensity of fluorescence of rhodamine-phalloidin (IFRF) conjugated with actin filaments] in dynamics of meiosis of BCB^- and BCB^+ porcine oocytes. Before IVM compact cumulus oocyte complexes (COCs) were incubated in BCB solution(13 μM) for 60 min. at 38.5°C in 5% CO_2. Then oocytes were divided into BCB^- (colorless cytoplasm) and BCB^+ (colored) oocytes. COCs were cultured in maturation medium (NCSU 23) supplemented with 10% follicle fluid (FF), 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. FF was collected from follicles with 3 - 6 mm in diameter. 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. For assessment of chromatin and IFRF fixed oocytes were incubated sequentially in rhodamine-phalloidin (RF, R415 Invitrogen, Moscow, Russia), 1 IU/ml, for 30 min to label actin. Then oocytes were incubated in 4',6-diamidino-2-phenylindole, 10 |μg/ml, for 10 min to label chromatin. Oocytes were examined using confocal laser scanning system Leica TCS SP5 with inverted fluorescent microscope. Diode 405 nm, argon 488 nm and helium-neon 543 nm laser lines were used for fluorochrome excitation. IFRF were expressed in arbitrary units. All chemicals used in this study, except for RF, were purchased from Sigma-Aldrich (Moscow, Russia). Data were analyzed by Student's t-test**.** Chromatin status and IFRF of 159 BCB^+ and 101 BCB^- oocytes (total 260 oocytes, in 3 replicates, 10-26 oocytes/group) were evaluated before and after 22 and 44 h of cultivation. Significant differences in IFRF of BCB^+ (33.2 ± 2.2) and BCB^- oocytes (42.6 ± 2.1) were identified before cultivation ($P < 0.05$). There were no differences between the IFRF in BCB^+ and BCB^- oocytes on metaphase-I stage(49.1 ± 6.9 and 51.2 ± 4.8, respectively). The decrease of IFRF in BCB+ oocytes was found after 44 h of IVM (49.1 ± 6.9 and 35.8 ± 6.3 ,respectively, $P < 0.01$). There were no differences between the IFRF in BCB^- oocytes on metaphase-I (51.2 ± 4.8) and methaphase II (49.1 ± 6.9) stages. Overall our data clearly showed that actin cytoskeleton actively involves in maturation of porcine oocytes in vitro. Features of the functional activity of actin cytoskeleton in BCB^+ and BCB^- oocytes during IVM have been identified.

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Modifying the fastness to age-related alterations of *in vitro* maturing bovine oocytes by luteotrophic factors and granulosa cells

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Keywords: progesterone, prolactin, oocyte aging.

The quality of mammalian oocytes determines their ability to embryonic development. Once the oocyte matures to the metaphase-II (M-II) stage, it undergoes accelerated senescence processes leading to an impairment of its quality. The objective of this work was to study effects of prolactin (PRL), progesterone (P4), and granulosa cells during the completion of in vitro maturation of bovine oocytes on their subsequent resistance to age-related functional alterations. Bovine cumulus-enclosed oocytes (CEOs) were cultured for 12 or 24 h in the first IVM medium (TCM 199 supplemented with 10% fetal calf serum (FCS), 10 μg/ml FSH, and 10 μg/ml LH) at 38.5°C and 5% CO_2. After the 12 h-culture, the CEOs were transferred to the second IVM medium (TCM 199 supplemented with 10% FCS) and matured for next 12 h in the absence and in the presence of granulosa cells (GCs) preliminary cultured under the same conditions for 12 h. The following additives to the second IVM medium were applied: (1) no additives (Control), (2) 25 ng/ml bovine PRL (Research Center for Endocrinology, Moscow, Russia), (3) 50 ng/ml PRL, and (4) 50 ng/ml P4. Then CEOs were cultured for additional 24 h in the aging medium (TCM 199 containing 10% FCS). At the end of culture, the state of the oocyte nuclear material was evaluated by the Tarkowski's method. Oocyte apoptosis was detected using the TUNEL kit (Roche, Indianapolis, USA). The data for the nuclear status (n=4, 80-91 oocytes per treatment) and apoptosis (n=5-6, 91-121 oocytes per treatment) were analyzed by ANOVA. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). During 24 h aging of CEOs matured in the first IVM medium without transfer, the rates of M-II oocytes with destructive changes of chromosomes (decondensation, adherence, clumping) and apoptotic oocytes rose ($P < 0.001$) from 28.6 ± 2.1% and 10.1 ± 1.4% (prior to aging) to 67.1 ± 2.0% and 24.3 ± 0.4%, respectively. Similar age-related increases in these rates were observed in case of CEOs matured during two-step culture. In the absence of GCs, P4 decreased the frequency of chromosome abnormalities in aged oocytes from 68.5 ± 1.9 (Control) to 51.2 ± 2.9% ($P < 0.01$), whereas PRL did not. Maturation of CEOs in the presence of GCs and PRL resulted in a reduction (at least $P < 0.01$) in the rate of oocytes with abnormal chromosome modifications following aging (from 67.9 ± 2.3% (Control) to 50.6 ± 3.9% (25 ng/ml of PRL) and 46.5 ± 5.0% (50 ng/ml of PRL)). By contrast, the addition of GCs to the second IVM medium abolished the positive effect of P4 ($P < 0.001$). Furthermore, P4 caused a decline ($P < 0.05$) in the rate of aged CEOs with apoptotic signs matured in the absence of GCs (from 24.3 ± 0.4% (Control) to 17.6 ± 1.6%). Thus, during the completion of bovine oocyte maturation, PRL and P4 can raise the subsequent resistance of aging ova to age-related changes in their quality, with GCs being able to modulate the hormonal effects. The study was supported by the Russian Science Foundation (project 16-16-10069).

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Effect of different estrous sheep serum batches on sperm capacitation and *in vitro* embryo development

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Keywords: sperm capacitation, *in vitro* fertilization, estrous sheep serum.

Nowadays, the estrous sheep serum (ESS) is the additive used to capacitate ram spermatozoa in *in vitro* fertilization systems. This non-defined substance is present during the co-incubation of spermatozoa and oocytes. The main problem of using non-defined substances is the need to test their validity and the effect of these substances on future embryo (García-Álvarez et al., Theriogenology 84:948-956 2015). The aim of this work was to assess the effect to capacitate thawed ram spermatozoa with different batches of estrous sheep serum on tyrosine phosphorylation, key event in capacitation, and on embryo yield. A pool of thawed semen from three rams was used to carry out the analysis of tyrosine phosphorylation by western blot and an *in vitro* fertilization trial. After discontinuous density gradient on Percoll, spermatozoa were incubated for 15 min in synthetic oviductal fluid (SOF) with 10% of three ESS batches (ESS1, ESS2 and ESS3). A negative control (SOF without ESS) was also used (NCap). The intensity signal of different bands was analysed with C-Digit® Blot Scanner from LI-COR and relativized to Tubulin. For the *in vitro* fertilization 395 oocytes were used and was performed according to García-Álvarez et al. (García-Álvarez et al., Theriogenology 84:948-956 2015). General lineal models were used with the following dependent variables: signal intensity, cleavage rate at 48 h post insemination (cleavage rate) and percentage of blastocyst at 9 days (embryo rate), and fixed variables: treatment (NCap, ESS1, ESS2 and ESS3) and replicate. There were no differences in the band intensity between treatment. However, cleavage and embryo rates were different for NCap and ESS1, ESS2 and ESS3 (0±9, 41±5, 46±6, 56±5, and 0±7, 27±5, 29±5, 31±5, respectively) but were similar between the ESS batches. In conclusion, the ESS batches do not influence sperm capacitation, cleavage and embryo rates although the presence of ESS is necessary to fertilize oocytes. Nevertheless, more studies of the quality of these embryos are necessary.

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Improvement of *in vitro* produced bovine embryo quality using Charcoal:Dextran Stripped Fetal Bovine Serum on culture media

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Keywords: CDS FBS, HI FBS, bovine embryo.

This study investigated the effect of Charcoal:Dextran Stripped fetal bovine serum (CDS FBS) and heat-inactivated FBS (HI FBS) in embryo culture medium (SOF-BE1 medium supplemented with 10% of serum) on their ability to support *in vitro* development of bovine embryos. Charcoal:Dextran treatment of FBS removes lipophilic chemicals, certain steroid hormones and certain growth factors. The developmental ability and quality of bovine embryos were determined by assessing their cell number, lipid content, mitochondrial activity, gene expression, and cryo-tolerance. The differences in embryo development (350 oocytes per each group were cultured in six replicates), integrated optical intensity, and expression levels of the various genes between experimental groups were analyzed by one-way ANOVA. The percentages of embryos that underwent cleavage and formed a blastocyst were significantly ($P < 0.05$) higher in medium containing CDS FBS than in medium containing HI FBS (42.84 ± 0.78% vs. 36.85 ± 0.89%, respectively). The total number of cells per day 8 blastocyst was ($P > 0.05$) higher in the CDS FBS group (208.40 ± 14.77) than in the HI FBS group (195.11 ± 19.15), however, this difference was non-significant. Furthermore, the beneficial effects of CDS FBS on embryos were associated with a significantly increased mitochondrial activity, as identified by MitoTracker Green, and reduced intracellular lipid content, as identified by Nile red staining, which increased their cryo-tolerance. The post-thaw survival rate of blastocysts was significantly ($P < 0.05$) higher in the CDS FBS than in the HI FBS group (85.33 ± 4.84% vs. 68.67 ± 1.20%). Quantitative real-time PCR showed that the mRNA levels of acyl-CoA synthetase long-chain family member 3, acyl-coenzyme A dehydrogenase long-chain, hydroxymethylglutaryl-CoA reductase, and insulin-like growth factor 2 receptor were significantly increased upon culture with CDS FBS. Moreover, the mRNA levels of sirtuin 1, superoxide dismutase 2, and anti-apoptotic associated gene B-cell lymphoma 2 in frozen-thawed blastocysts were significantly ($P < 0.05$) higher in the CDS FBS group than in the HI FBS group, however, the mRNA level of the pro-apoptotic gene BCL2-associated X protein was significantly reduced. Taken together, these data suggest that supplementation of medium with CDS FBS improves in vitro bovine embryo developmental competence and cryo-tolerance. This work was partly supported by a grant from the Next-Generation BioGreen 21 Program (No. PJ01107703) and IPET (Grant no. 315017-5, 117029-3).

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Novel approach for the measuring mitochondrial function in bovine oocytes and embryos

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Keywords: oxygen consumption, oocytes, metabolism.

Oxygen consumption is an established marker for cellular energy metabolism and an indicator of mitochondrial function. In reproductive biology, it has been correlated to a range of outcomes including oocyte viability and maturation, embryo development, implantation potential and pregnancy rate. However, measuring oxygen consumption is technically challenging, requiring specialist equipment. The recent availability of Seahorse Bioanalysers has transformed the study of cellular metabolism in a range of systems, however to date, this technology has not been applied to oocytes and embryos. We have therefore examined whether Seahorse XFp is capable of measuring oxygen consumption of small groups of oocytes and embryos. Bovine oocytes were collected from abattoir-derived ovaries and cultured overnight in maturation media (nutrient and hormone-supplemented M199). Oocytes were either allowed to mature or treated with cycloheximide to maintain their immature state. 2-cell embryos generated by IVF were selected after culture for ~24 hours in synthetic oviduct fluid (SOF). Media used was made up in-house as has been reported previously (Guerif et al., PloS One, Volume 8, e67834, 2013). Oocytes or embryos were loaded into Seahorse XFp bioanalyser plates in groups of 6 and oxygen consumption rate (OCR) was measured non-invasively. To further probe the constituents of oxidative function in bovine oocytes, mitochondrial uncoupler FCCP, and electron transport chain inhibitors oligomycin and Antimycin A/rotenone were serially injected (Sigma Aldrich). Assays were repeated on three independent occasions. Data was analysed using one way ANOVA with Tukey's post-hoc. Using this approach, we were able to generate reproducible OCR values for bovine oocytes and embryos. 2-cell embryos were significantly different ($P<0.01$) to germinal vesicle (GV) stage and metaphase-II (met-II) stage oocytes – 0.62 ± 0.15 pmol/min/embryo, compared to 2.36 ± 0.22 and 1.83 ± 0.31 pmol/min/oocyte respectively (mean ± SEM). Crucially, fertilisation rates for oocytes having undertaken the assay were not significantly different to controls ($P>0.05$). The response to mitochondrial inhibitors, shown in real-time, indicated that the approximately 60% of oocyte OCR was coupled to ATP synthesis, 20% was non-mitochondrial with the remaining being proton leak. Furthermore, oocytes have the capacity to increase OCR by approximately 60% spare capacity. These data demonstrate the use of the Agilent Seahorse XFp as a technique for the direct assessment of mitochondrial function in bovine oocytes and embryos. Importantly, we demonstrate that oocytes are competent to undergo fertilisation after this assay, indicating the non-invasive nature of the test. Compared to previously applied assays for oxygen consumption, Seahorse is fast, simple and automated, allowing investigations of higher throughput. With increasing recognition of the critical role mitochondria play in supporting healthy reproduction; this tool facilitates investigation into mitochondrial function which has extensive scope for applications within reproductive biology.

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Bovine embryos release extracellular vesicles into the medium during group culture *in vitro*

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Keywords: embryo-embryo communication, extracellular vesicles, embryo culture.

Efficient communication between cells and tissues is paramount in many physiological process, including embryo development. Inside the genital tract, embryos usually communicate with the mother and vice versa through autocrine, paracrine and endocrine signaling. From our previous research (Wydooghe et al. Reprod. Fertil. Dev, 26, 115, 2013) it has been clearly demonstrated that, in the absence of maternal genital tract, preimplantation embryos cultured in group are able to promote their own development *in vitro* by the production of autocrine embryotropins. Recent studies indicate that among autocrine secreted factors extracellular vesicles play a prominent role in communication. Extracellular vesicles are membrane bound vesicles that are found in biological fluids and in culture media conditioned by embryos or cells. They carry and transfer regulatory molecules, such as microRNAs, mRNAs, lipids and proteins. Here we show that preimplantation bovine embryos cultured in group can release extracellular vesicles into the medium, as novel way of embryo communication. The aim of the current study is to standardize a protocol for isolation and quantification of extracellular vesicles from culture medium conditioned by bovine embryos. Since BSA(Sigma A9647) may contain extracellular vesicles, for optimization of this protocol, bovine presumed zygotes (n = 1140, 4 replicates) produced *in vitro* were allocated to two culture media (SOF with insulin, transferrin and selenium supplemented with either 0.4% BSA (Sigma A9647) or with 0.1 % PVP(Sigma P5288)) and were cultured until Day 8. Media conditioned by embryos were pooled at day 8 until 1ml was obtained, and subjected to density gradient ultracentrifugation (Van Deun et al., J. Extracell. Vesicles. 3, 2014) to extract extracellular vesicles. Extracted suspension with extracellular vesicles was analyzed with Nano particle tracking for quantification. For identification, negative staining electron microscopy was performed, and specific antibodies CD9 (CST), CD63(Serotec) and TSG101(Abcam) were tested for further confirmation of extracellular vesicles presence in the extracted suspension. Blastocyst development rate on day 8, was analyzed by using Student t test (Statistical Analysis System (SAS) for Windows) had showed no significant difference between both media's (40±3.43 % vs 38.64±2.88 %; in SOF+BSA vs SOF+PVP respectively). Results obtained from extracellular vesicles quantification and identification analysis provided evidence that bovine embryos can release extracellular vesicles with a size ranging from 40~200nm into the culture medium. The concentration of extracellular vesicles extracted from 1ml of conditioned medium was 9.18×10^7 +/- 4.52×10^7 particles/ml. Further experiments will be performed to extract a higher concentration of extracellular vesicles from a limited amount of medium conditioned by embryos, by following different isolation techniques, such as size exclusion chromatography and ultra-centrifugation.

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The effect of diet on fatty acid composition of elongated bovine conceptuses

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Keywords: dairy cattle, CLA, milk fat depression.

Conceptus elongation is crucial for establishment of pregnancy in ruminants. During the elongation step, endometrium secreted lipids are required as a source of energy and for the remodeling and proliferation of cellular membranes (Ribeiro et al., Reprod 2016;152:R115-R126). The present study investigated the effects of diets designed to cause 15 % reduction in milk fat synthesis (milk fat depression) on fatty acid (FA) composition of elongated bovine conceptuses. The day-14 conceptuses were collected from 22 Nordic Red multiparous dairy cows during two sequential indoor housing periods. Treatments comprised a grass silage based basal diet (CO; 10 cows), basal diet supplemented with 12 g/d of rumen protected *trans*-10, *cis*-12 CLA (Lutrell Pure, BASF, Germany) (CLA; 8 cows), and grass silage based diet containing high-starch concentrate components and supplemented with 26.7 g/kg diet dry matter of sunflower oil and 13.3 g/kg diet dry matter of fish oil (MFD; 4 cows). CLA supplement was mixed in total mixed ration and administered in two equal proportions per day. Oil supplements replaced concentrate ingredients. Cows were randomly allocated to the treatments immediately after parturitions. The diets were total mixed rations with 55:45 forage:concentrate ratio on dry matter basis. In total of 45, 35 and 13 conceptuses having visible embryonic discs were used after recovery from the superovulated CO, CLA and MFD donors 130 days after parturitions, respectively. Lipids were extracted separately from each cryopreserved conceptus using a mixture of hexane and 2-propanol (3:2, vol/vol). FA were transesterified to methyl esters using methanolic sodium methoxide and analyzed with a gas chromatograph equipped with a flame ionization detector (Shingfield et al., Anim Sci 2003;77:165–179). Data were analyzed using linear mixed models with MIXED procedure in SAS 9.4. The proportions of the most abundant FA in conceptuses, *cis*-9 18:1 (30-32 g/100g FA, %), 16:0 (25-26 %), and 18:0 (12-14 %), did not differ between treatments ($P > 0.10$). In addition, total proportions of *cis* unsaturated FA (53-54 %) and saturated FA (43-45 %) and ratio of *cis* unsaturated FA to saturated FA was not different among treatments ($P > 0.10$). CLA had no effect on the conceptus' FA composition compared with CO. However, MFD induced higher ($P < 0.01$) 22:6n-3, and lower ($P < 0.05$) 22:4n-6 and 22:5n-6 proportions compared with CLA and CO. The proportion of *cis*-12 18:1 and *trans*-9, *cis*-12 18:2, which are biohydrogenation products of 18:2n-6 found in rich amounts in sunflower oil, tended to be higher ($P < 0.10$) in MFD compared with other treatments. In conclusion, although CLA and MDF caused changes in lipogenesis in the mammary gland and milk fat depression, a substantial effect on the FA composition of conceptuses was not observed. However, unsaturated FA deriving from the MFD diet and metabolized in the rumen biohydrogenation processes had a specific impact on conceptus FA profiles during elongation stage. Acknowledgements: This research was supported by the Ministry of Agriculture and Forestry (Grant No. 1834/312/2014).

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Expression profile of genes involved in sex determination in cattle

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Keywords: sex determination, cattle, gonad.

Sex determination is the process by which a bipotential gonad develops into a testis or ovary depending on the genetic background of the individual. There is a paucity of information about this process in large mammalian species. Bovine *SOX9* (a critical gene for sex determination in mice and humans) has lost the two transactivation motifs that are essential for sex determination in mammals; therefore, an alternative sex determination pathway could be responsible for sex determination in cattle. In order to clarify the genes involved in gonad differentiation in cattle we performed quantitative analysis of RNA expression in the genital ridges of bovine foetuses collected at the abattoir. The age in days from conception was estimated based on the crown-rump length (CRL). We collected 14 female foetuses ranging in CRL from 18 mm (Day 38 of development) to 57 mm (Day 59) and 19 male foetuses ranging from 13 mm (Day 33) to 48 mm (Day 56). The sex of the embryos was determined by PCR with primers for bovine amelogenin. RNA was extracted and converted to cDNA using a reverse transcription kit (Applied Biosystems, Carlsbad, CA). All qPCR reactions were carried out using a PCR mix (GoTaq® qPCR Master Mix, Promega Corporation, Madison, USA) containing the primers selected for the bovine genes *SF1* and *WT1* (related with gonadal formation)*; FOG2*, *GATA4*, *SOX9*, *SRY*, *DMRT1* (involved in sex determination and testis development); *WNT4*, *FOXL2* (participating in ovary formation and maintenance, respectively); and *ZRSRY2*, *SOX8*, *SOX10* (candidates genes potentially involved in testis determination in cattle). The reference gene used was H2AFZ. In addition, the location of the primordial germ cells (PGC) was evaluated by immunohistochemistry to identify testis and ovary formation differences between the sexes after SRY peaks. Genital ridges were fixed and stored in Bouin's solution for immunohistochemistry of cell marker OCT4. We found that *SRY* expression peaked at a CRL of 18 mm (Day 38). We detected expression of *SOX8* and *SOX10* in male foetuses after the SRY peak (earlier than observed in mice and humans), and *ZRSR2Y* (a splicing factor related to RNA processing and RNA splicing) expression along all the stages analysed showing an increasing pattern from Day 33 to Day 56. Regarding immunohistochemistry, we identified that PGC follow two distinct patterns in males and females. Before SRY peak, PGC localize along the genital ridges of both sexes. After SRY peak, testis cords begin to be distinguishable at a CRL of 25 mm (Day 42) in males, with one to three PGC within each of the developing tubules. In the case of females, PGC tend to distribute along the periphery of the developing ovary at a CRL of 36 mm. Overall, these results indicate that sex determination in bovine genital ridges present characteristic features with SOX8 and SOX10 showing early expression after SRY peak, and ZRSR2Y as a splicing factor that could be involved in sex determination.

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Nuclei number and DNA fragmentation in pig embryos derived from IVF, *in vivo*-IVC and *in vivo*-derived blastocysts evaluated by TUNEL assay

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Keywords: pig, embryos, quality.

Embryo quality is assessed on the basis of the rate of development, morphology, number of cell nuclei and the degree of apoptosis of the embryonic DNA. Detection of apoptosis in embryos is possible due to the occurrence of DNA fragmentation, the degree of which can be determined using the TUNEL assay. The aim of the study was to compare the quality of pig embryos obtained after IVF (IVP), *in vivo*-IVC (*in vivo* zygote and *in vitro* culture) with *in vivo*-derived blastocysts (control) by TUNEL assay. IVP embryos were obtained from *in vitro*-matured, *in vitro* fertilized oocytes. The putative zygotes were cultured in NCSU-23 medium at 39°C and 5% CO_2 in the air up to the blastocyst stage. *In vivo*-IVC zygotes were obtained surgically from superovulated and inseminated donor gilts and cultured in NCSU-23 medium up to the blastocyst stage. *In vivo*-derived blastocysts were obtained surgically on the sixth day after insemination from superovulated and inseminated donor gilts by flushing the uterus with PBS-BSA solution. The IVP, *in vivo*-IVC and *in vivo*-derived blastocysts were subjected to TUNEL assay according to the manufacture protocol (TUNEL reagent In Situ Cell Detection Kit, Roche Diagnostic, Germany). The analysis was carried out under an epifluorescence microscope using the following filters: 520 nm (TUNEL) and 358-461 (DAPI). Statistical analysis was performed using the t-test. It was observed that the mean number of cell nuclei was statistically significantly higher in *in vivo* embryos compared to *in vivo*-IVC and IVP embryos (106.47; 39.20 and 38.73; respectively, $P<0.01$). In turn, the mean number of apoptotic nuclei was significantly higher in embryos derived *in vivo*-IVC compared to IVP and *in vivo* embryos (2.56; 1.63 and 0.06 respectively, $P<0.01$). The TUNEL index was 4.20% for IVP, 0.06% for *in vivo*-derived blastocysts and 6.53% for *in vivo*-IVC blastocysts. The study showed that quality of IVP and *in vivo*-IVC embryos was lower compared to the quality of embryos derived *in vivo*. The quality of the embryos thus obtained is mainly affected by the *in vitro* culture conditions.

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Investigating the impact of hyperglycaemia on bovine oviduct epithelial cell physiology and secretions *in vitro*

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Keywords: oviduct, hyperglycaemia, epithelium.

A key role of the oviduct, or Fallopian tube, is the creation of the environment where fundamental developmental processes take place, including gamete activation, fertilisation and early embryo development. Previous studies have partially determined the composition of oviduct fluid. However, the impact of maternal physiology on the oviduct environment is unknown. The aim of this study was to investigate the impact of a hyperglycaemic challenge on the physiology of oviduct epithelial cells in vitro as well as the biochemical and physical properties of oviduct-derived fluid, using an air:liquid model of the oviduct. Bovine oviduct epithelial cells, harvested from slaughterhouse-derived tissues (mainly stage II reproductive tracts), were cultured in DMEM-F12, at 39oC and 5%CO2 for 6 days. Cell identity was confirmed using confocal and optical microscopy. The cells were grown to confluence on a permeable membrane, allowing selective transportation of nutrients between apical and basal chamber. TransEpithelial Electrical Resistance (TEER) measurements, >700Ohm.cm2 were used to indicate the barrier properties of the epithelial monolayer(n=4). This was independently confirmed by observing that fluorescein was unable to cross the monolayer(n=3), when the cells were determined confluent by TEER measurements. Once confluence was achieved, the apical medium was discarded and cells cultured in an air:liquid interphase, mimicking the in vivo environment. After 24h, a thin film of fluid accumulated, which was collected for biochemical analysis. In Experiment 1, physiological (7.3mM) and hyperglycaemic (8.5mM,11mM) concentrations of glucose were added together with 20ng/ml of insulin to the basal compartment for 24h. In Experiment 2, cells were exposed to the same conditions but for 7 days. Data were analysed using Kruskal-Wallis test with Dunn's post-hoc. Chemicals and consumables were used as previously (Simintiras et al, Reproduction, 153,23–33,2017). Our data revealed that acute hyperglycaemia in the basolateral compartment did not change the luminal concentrations of glucose, pyruvate or lactate. However, the presence of insulin reduced glucose in the lumen when cells were exposed to hyperglycaemia. By contact, 7-day basolateral exposure to hyperglycaemia in the absence of insulin increased luminal concentrations of glucose (1.09mM for normoglycaemia compared to 8.9mM for chronic hyperglycaemia). Notably, the presence of insulin reduced the volumes of oviduct-derived fluid (6.24µl in hyperglycaemia compared to 75.5µl for normoglycaemia ($P<0.05$). Using an in vitro oviduct model we have shown that long term exposure to hyperglycaemia induces glucose transport in oviduct secretions and that insulin appears to reduce fluid flow across the oviduct monolayer. Future work will focus on investigating differences in gene expression in response to hyperglycaemia, as well as a detailed evaluation of how insulin affects ion transport. Furthermore, we will determine the impact of hyperglycaemic-conditioned oviduct fluid on gamete maturation and early embryo development.

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Effect of oxygen tension on the development of *in vitro* embryos from Iberian red deer (*Cervus elaphus hispanicus*)

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Keywords: oxygen tension, iberian red deer, *in vitro* fertilization.

Culture conditions during *in vitro* oocyte maturation and fertilization, such as exposure to different oxygen concentrations, have been shown to affect in the developmental ability and the generation of reactive oxygen species (ROS). Low oxygen concentrations may significantly improve the developmental potential of cleavage stage embryos, thus resulting in a positive effect on subsequent blastocyst formation in different species (Leoni et al., Reprod Domest Anim 42(3):299–304. 2007). With the aim to improve the *in vitro* production of embryos in the Iberian red deer (*Cervus elaphus hispanicus*), we evaluated the influence of two oxygen tensions (5 and 21%) during *in vitro* maturation (M) and fertilization (F) on developed blastocysts. The *in vitro* embryo production was performed as García-Álvarez et al., Theriogenology 75:65-72, 2011 and Berg et al., Anim Reprod Sci 70:85-98. 2002. Similarly, we analysed differences in gene expression of the resulting expanded blastocysts. To assess embryo production, a total of 588 COCs were divided into four experimental groups that were evaluated according to the oxygen tension used (M5F5, M5F21, M21F5 and M21F21). Relative poly(A) mRNA abundance of *GAPDH*, *G6PH*, *HPRT*, *SOD2*, *BAX*, *SHC1*, *AKR1B*, *PLAC8*, *GJA1* and *SOX2* was analyzed using quantitative real-time RT-PCR (qRT-PCR). General linear models were used with the independent variable being percentage of total blastocysts at 9 days (embryo rate=96 blastocysts); and the fixed variables being treatment (M5F5, M5F21, M21F5 and M21F21) and replicate (n=7). Additionally, relative mRNA abundance differences in blastocysts were analyzed by one way ANOVA. Results showed that regardless of the oxygen concentration, blastocyst rates did not differ ($P \geq 0.05$) (M5F5=21.98±6.26; M5F21=12.46±5.78; M21F5=18.21±2.90; M21F21=20.54±5.75). With regard to gene expression, *SOD2* was up-regulated ($P<0.05$) in oocytes matured in low oxygen, independently of the tension used during fertilization, whereas *SOX2* was down-regulated ($P<0.05$) in oocytes that were also matured in low oxygen but fertilized in high oxygen tension ($P<0.05$). Likewise, *AKR1B* and *PLAC8* were up-regulated ($P<0.05$) when oocytes were matured and fertilized under high tensions. To our knowledge, this is the first study that demonstrates that Iberian red deer embryos can be produced *in vitro* using different oxygen tensions. Although the four groups compared do not reflect significant differences in terms of embryo production, the use of different oxygen tensions during *in vitro* maturation and fertilization significantly alters the expression of genes related to oxidative stress (*SOD2*), implantation (*AKR1B1* and *PLAC8*) and transcription factors involved in the regulation of embryonic development and determination of cell fate (*SOX2*). In conclusion, both oxygen tensions (5 and 21%) result in similar embryonic development and therefore are feasible for *in vitro* production of Iberian red deer embryos, but more studies are necessary to determine blastocyst quality. Supported by MINECO (AGL2013-48421-R).

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Regulatory actions of progesterone and luteotrophic hormones on bovine oocyte apoptosis during the terminal phase of *in vitro* maturation

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Keywords: progesterone, luteotrophic hormones, oocyte apoptosis.

The currently available evidence points out the cumulus-derived progesterone (P4) as a key antiapoptotic signal involved in maintaining the bovine oocyte viability during in vitro maturation (O'Shea et al., Biol Reprod, 89:146, 2013). However, effects of exogenous P4 on the oocyte quality are not quite clear. The aim of the present research was to compare actions of P4 and two luteotropic hormones, prolactin (PRL) and LH, on apoptosis of bovine oocytes during the second phase of in vitro maturation (from M-I to M-II). Bovine cumulus-oocyte complexes (COCs) were cultured for 12 h in TCM 199 containing 10% fetal calf serum (FCS), 10 μg/ml of porcine FSH, and 10 μg/ml of ovine LH at 38.5°C and 5% CO_2. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Then COCs were transferred to the following culture systems: (1) TCM 199 containing 10% FCS (Control 1) and (2) a monolayer of granulosa cells (GCs) precultured for 12 h in TCM 199 containing 10% FCS (Control 2). Just before the oocyte transfer, the medium of experimental groups was supplemented with either 50 ng/ml of P4 or 50 ng/ml of bovine PRL (Research Center for Endocrinology, Moscow, Russia) or 10 μg/ml of ovine LH. At the end of culture, the nuclear status of oocytes was assessed by staining with DAPI. Oocyte apoptosis was detected using the TUNEL kit (Roche, Indianapolis, USA). The content of P4 and estradiol-17β (E2) in culture media was determined by ELISA. All data (n=5, 87-99 oocytes per treatment) were analyzed by ANOVA, with percentage data being arcsine transformed. After 24 h of culture, the rate of M-II oocytes was similar in the compared groups and reached 80.3-89.2%. The addition of P4 to the control medium of both systems resulted in the reduction ($P<0.05$) of the apoptosis frequency in matured oocytes from 11.7±1.2 to 5.9±1.7% (System 1) and from 13.8±1.6 to 7.2±0.9% (System 2). In the absence of GCs, PRL and LH did not affect oocyte apoptosis. When COCs were cocultured with GCs, the apoptosis rates increased ($P<0.05$) from 9.4±1.6 (without GCs) to 16.3±1.8% for the PRL groups and from 13.0±1.5 (without GCs) to 17.6±2.2% for the LH groups. Meanwhile, in the presence of GCs, these rates were higher than that for the P4 group ($P<0.01$). At the end of oocyte culture in both systems, the content of P4 in the medium was 1.2-1.3 times lower ($P<0.05$) in groups treated with PRL or LH than in the group treated with P4, whereas the content of E2 did not differ between groups tested. Furthermore, concentrations of P4 and E2 were increased 1.2-1.3 times (at least $P<0.05$) in the presence of GCs regardless of the hormonal treatment. Our findings indicate that exogenous P4 can exert granulosa-independent antiapoptotic action on bovine oocytes during the second phase of in vitro maturation. At the same time granulosa cells are able to cause proapoptotic effects of PRL and LH on the oocytes that complete maturation. This research was supported by the Russian Science Foundation (project 16-16-10069).

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Effect of prolactin and dithiothreitol during prolonged culture of aging oocytes on the development potential of parthenogenetic bovine embryos

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Keywords: oocyte aging. parthenogenetic embryos.

The evaluation of factors responsible for the protection of the oocytes attained the metaphase-II stage from aging is important for successful in vitro embryo development. The aim of the present research was to study dose-dependent effects of two potential regulators of oocyte quality, prolactin (PRL) and dithiothreitol (DTT), during the prolonged culture of bovine oocytes on their developmental potential after artificial activation. Slaughterhouse-derived cumulus-oocyte complexes (COCs) were matured for 22 h in TCM-199 supplemented with 10 % fetal calf serum (FCS), 0.2 mM sodium pyruvate, 10 μg mL^{-1} porcine FSH, and 10 μg mL^{-1} ovine LH. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). After 22 h maturation, the part of COCs were transferred to the fresh medium consisting of TCM 199 supplemented with 10 % FCS and cultured for 10 h in the absence (Control) or in presence of bovine PRL (20 and 50 ng mL^{-1}; Research Center for Endocrinology, Moscow, Russia) or DTT (2.5, 5 and 10 μM). After maturation (22 h) or the prolonged culture (10 h), oocytes were activated by culturing in 5 μM ionomycin solution during for 5 min followed by 4 h in 2 mM 6-dimethylaminopurine. Activated oocytes were cultured in CR1aa medium (Rosenkrans, First, J Anim Sci 1994, 72:434-7) until Day 5 and then transferred to the same medium supplemented with 5 % FCS and cultured up to Day 7. All the cultures were performed in at 38.5°C and 5% CO_2 in humidified air. At Days 2 and 7 after activation, the cleavage and blastocyst rates were determined. In addition, obtained blastocysts were fixed with 4% paraformaldehyde, and the total cell number was determined by DAPI staining. The data from 4 replicates (111-122 oocytes per treatment) were analyzed by ANOVA. For oocytes activated just after IVM, the cleavage and blastocyst rates, and total blastocyst cell number were 74.1±3.5 and 20.6±2.8 %, and 54,0±1,8, respectively. The prolonged culture of matured COCs in the aging medium (10 h) (Control) had no effect on the cleavage rate (74.7±2.9 %) and the total number of cells in embryos (49.1±2.0), but caused the blastocyst yield to decline to 9.8±1.2 % ($p<0.05$). At the same time, the addition of both PRL (50 ng mL^{-1}) and DTT (5 μM) to the aging medium raised the blastocyst rate to 18.0±3.1 and 18.8±2.5 % ($p<0.05$), respectively. Cleavage rates of aging oocytes after their activation and total cell number in blastocyst produced from aging oocytes were unaffected by both PRL and DTT (except DTT 10 μM group). In the case of DTT 10 μM, the blastocyst rate and total blastocyst cell number (9.6±1.3 % and 35.4±1.1, respectively) was lower than in the DTT 5 μM group ($P<0.05$). Thus, PRL and DTT are able to maintain competence for parthenogenetic development of bovine COC during their prolonged in vitro culture.

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A279E Embryology, developmental biology, and physiology of reproduction

Initial characterization of bovine embryos developing at the air-liquid interphase on oviductal epithelial cells

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Keywords: Air-liquid interphase, oviductal epithelial cells, embryo development.

Air-liquid interphase cultures of bovine oviductal epithelial cells (ALI-BOEC) have recently been reported to support embryo development in co-culture up to the blastocyst stage without the addition of embryo culture medium. To initially characterize such ALI-BOEC produced embryos, we assessed the expression of 41 target genes in 8-cell embryos and blastocysts. For comparison, we analyzed embryos produced in standard IVP media (IVP-S) and in a commercial serum-free media suit (IVP-SF; IVF Bioscience, Falmouth, UK). A total of fifteen 8-cell embryos (randomly divided into 3 pools of 5 embryos) and six single blastocysts were collected under each culture condition. Gene expression was analyzed by means of a 48.48 Dynamic Array™ on a Biomark HD instrument. To identify genes differentially expressed in 8-cell embryos and blastocysts, we applied one-way ANOVA with Tukey post-hoc test in SPSS. Furthermore, we re-analyzed previously published transcriptomics data from *in vivo* embryos (GSE12327). The cleavage rate in ALI-BOEC co-culture (70.71%) was comparable to the standard IVP procedure (74.75%), and lower than the cleavage rate reached with the commercial media suit (90.79%). However, the blastocyst rate in ALI-BOEC co-culture (9.1%) was much lower than in either IVP-S (33.1%) or the commercial IVP-SF system (54.7%). Re-analysis of the *in vivo* data set revealed that sixteen of the chosen target genes were significantly regulated between the 8-cell and blastocyst stage embryos *in vivo*. The *in vitro* embryos showed expression patterns similar to the *in vivo* embryos. The culture conditions lead to differential gene expression in both 8-cell embryos (CDH1, NOS2, OVGP1, APEX1, REX1, PLAGL, BAX, SREBP1, SMPD2) and blastocysts (CCL26, CDH1, NID2, IFNAR1, SLC2A5, SREBP1, SERPINE1, LDLR, CYP51A1), respectively. Five of the genes differentially expressed in blastocysts from different culture conditions (LDLR, CDH1, NID2, SLC2A5 and CYP51A1) were previously reported to also be differentially expressed between *in vivo* and *in vitro* blastocysts. Embryos produced in the ALI-BOEC co-culture system followed the *in vivo* expression pattern for all five genes. The present study confirmed that the ALI-BOEC co-culture system is much less efficient in supporting blastocyst formation than conventional IVP procedures. Given the lack of a direct comparison to *in vivo* embryos, interpretation of the biological relevance of the differentially expressed genes warrants caution. However, our results indicate that blastocysts produced on ALI-BOEC may have an improved *in vivo*-like gene expression signature. The establishment of a sequential culture system of oviductal and uterine epithelial cells including a hormonal stimulation protocol might further increase the efficiency of the co-culture both quantitatively and qualitatively. Acknowledgments: This study was partly funded by the Schweizerischer Nationalfonds (SNF). Data analyzed in this paper were generated in collaboration with the Genetic Diversity Centre (GDC), ETH Zurich.

A280E Embryology, developmental biology, and physiology of reproduction

Maternal metabolic disorders and early embryonic loss: pathways to bridge the gap between bovine embryo quality and endometrial receptivity

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Keywords: nutrient sensing, blastomere differentiation, BEEC responses.

The pre-implantation embryo is very sensitive to perturbations in its micro-environment and, therefore, a tight regulation of the embryonic milieu is essential. Such an environment is not assured in females suffering metabolic disorders. Our previous data show that altered nutrient abundance in the embryonic micro-environment results in suboptimal embryonic cell differentiation patterns. Here, we hypothesize that suboptimal nutrient conditions during embryo culture can affect the blastocyst's capacity to participate in the first maternal-embryonic interactions. Earliest preimplantation phases of embryo development were studied as 'window' for nutrient sensitive manipulations. Embryos (4 repeats; 890 zygotes) were cultured during the first 4 days after fertilization (p.i.) in distinct nutrient conditions: [control] based on serum-free SOF medium; [HIGHGLUC] with 3.5mM glucose; [LOWAA] with 10% lower amino acid concentrations as presented in control. At morula stage, embryos were transferred to monolayers of bovine luminal epithelial endometrial cells (BEEC; subculture 1), in SOF medium + 5% serum, till D8 p.i.. In D8 blastocysts, mRNA expression of 12 genes involved in nutrient sensing, pluripotency and differentiation was analyzed by qRT-PCR. Differently expressed genes (DEG) were identified using (mixed model) ANOVA. Using NGS, transcriptomes of BEEC (4 repeats) exposed to distinct groups of embryos were sequenced and data were normalized by EdgeR. Blastocysts originating from HIGHGLUC morulae displayed a tendency for increased transcript levels of *PDK1* ($P=0.075$), a key gene in nutrient sensing regulation. Also a down-regulated expression of the pluripotency marker, *OCT4* ($P=0.002$), was observed compared to controls. Transcriptome reaction of BEEC exposed to the HIGHGLUC embryos was rather limited. Only 27 DEG genes were identified, of which 20 down- and 7 up-regulated in BEEC exposed to HIGHGLUC embryos compared to control embryos ($Padj<0.1$). Enriched genes involved endoplasmic reticulum activities, whereas cell-cell signalling pathways were down-regulated. Blastocysts from LOWAA conditions showed tendencies ($P\leq0.1$) for decreased transcript levels of *SIRT1*, *mTOR*, *GLUT1* and *LDHA*, all involved in mTOR pathways. Also a down-regulated mRNA expression was observed for *OCT4* ($P<0.0001$) and *SOX2* ($P<0.1$), both genes involved in pluripotency, and for *ITGB5* ($P<0.05$) and *CTNN1* ($P\leq0.1$), two blastomere differentiation markers. BEEC exposed to LOWAA embryos revealed 120 DEG compared to BEEC exposed to controls ($Padj<0.1$). Here, 63 of the 120 DEG were down- and 57 were up-regulated in the LOWAA condition. Up-regulated genes involved transcription regulation and down-regulated genes concerned inhibition of both Notch and immune responses. Overall, suboptimal metabolite conditions during the first 4 days of embryo culture can impact on resultant blastocyst cell proliferation and differentiation pathways. Furthermore, BEEC genes were differently regulated when placed in contact with the three distinct groups of embryos.