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Oviduct extracellular vesicles: a new strategy to optimize porcine *in vitro* embryo production

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Keywords: oviduct extracellular vesicles, embryo development, porcine IVP.

Oviduct extracellular vesicles (EVs) have been identified as important components of oviductal fluid (OF) and as key modulators of embryo-maternal communication. In an attempt to optimize porcine *in vitro* embryo production (IVP) outcomes by mimicking the maternal environment, we evaluated the effect of porcine oviduct EVs supplementation during *in vitro* culture (IVC) on porcine embryo development rate and quality. The effect of OF supplementation (10%) during IVC was also tested. Thus, five different treatments were used: Control (without supplementation); OF 0-2 (during the first 2 days of IVC); OF 0-7 (during 7 days of IVC); EVs 0-2 and EVs 0-7. Porcine oviducts were collected at local abattoir and flushed with PBS (Sigma, Saint-Louis, USA). EVs were isolated by ultracentrifugation and stored at -80°C until use (3 EVs/OF pools of 4 oviducts pairs each, from gilts at late follicular stage of estrous cycle). EVs protein concentration were measured (21.8-28.3 mg/mL) and diluted in IVC medium (NCSU-23; 0.2 mg/mL). *In vitro* matured and fertilized presumptive zygotes were cultured under mineral oil, 5% CO_2 and 38,8ºC, as detailed above (6 replicates) (Almiñana C., Theriogenology, 64, 1783–1796, 2005). Cleavage rates were evaluated at day 2 and blastocyst rates at days 5, 6 and 7 after fertilization. Embryo quality was assessed by hatching rates and number of cells/blastocyst at day 7. Data were analyzed by one-way ANOVA or Kruskall-Wallis test and expressed as mean ± SEM. EVs treatments (0-2 and 0-7) had a positive effect on cleavage rates being significantly higher than control ($P < 0.05$). Furthermore, EVs 0-2 treatment increased significantly cleavage rates compared to both OF treatments (0-2 and 0-7) ($P < 0.05$) (Cleavage rates: 44.4 ± 0.8; 46.4 ± 1.5; 45.7 ± 1.5; 52.1 ± 2.5; 51.1 ± 2.4; for Control, OF 0-2, OF 0-7, EVs 0-2 and EVs 0-7). Similarly, EVs 0-2 improved blastocyst rates over time, being significantly different to both OF treatments at day 7 ($P < 0.05$). Moreover, EVs 0-2 showed a tendency to increase blastocyst rates on day 5, 6 and 7 of IVC compared to EVs 0-7 ($P = 0.052$-0.08) (Blastocyst rates on day 5: 8.9 ± 0.8; 11.7 ± 2.6; 3.8 ± 0.4; 13.4 ± 2.7; 7.6 ± 2.2; on day 6: 16 ± 1.5; 15 ± 1.9; 9 ± 1.3; 23 ± 4.1; 16 ± 3.6 and on day 7: 21 ± 1.3; 19 ± 1.7; 10 ± 1.3; 29 ± 2.2; 20 ± 2.2; for Control, OF 0-2, OF 0-7, EVs 0-2 and EVs 0-7). However, the use of OF 0-7 decreased dramatically the blastocyst rates on day 5, 6 and 7 of embryo development ($P < 0.05$). There were no differences on hatching rates for any of the treatments tested. However, EVs 0-2 and OF 0-2 treatments increased significantly the number of cells/blastocyst when compared to the same treatments during longer IVC times (38.8 ± 3.8; 44.3 ± 4.2; 32.8 ± 2.2; 43.2 ± 3.1; 32.8 ± 1.4; for Control, OF 0-2, OF 0-7; EVs 0-2 and EVs 0-7). In conclusion, these results show that oviduct EVs supplementation during the first two days of development improves blastocyst yield and quality, suggesting that EVs could be a new strategy to improve porcine IVP. *Supported by* Ciências sem fronteiras fellowship n° 205593/2014-3 and INRA-CI-PHASE funds.

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Physiological concentrations of steroid hormones during *in vitro* culture changed lipid composition and improved cryosurvival of bovine embryos

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Keywords: embryos, steroid hormones, lipids.

Steroid hormones are highly concentrated in the bovine oviductal fluid in the post-ovulatory period, during fertilization and early embryo development. The objective of this study was to evaluate the effect of progesterone (P4), estradiol (E2) and cortisol (CO) at physiological concentrations on bovine embryo development rates and quality *in vitro*. Bovine oocytes were collected in a local slaughterhouse, matured and fertilized *in vitro*. Presumptive zygotes were cultured during 8 days in 500 µl of synthetic oviductal fluid supplemented with 55 ng/mL P4, 120 pg/mL E2, 40 ng/ml CO or their combination (P4/E2/CO) at the same concentrations, i.e. mean concentrations previously measured by mass spectrometry (MS) in post-ovulatory oviductal fluids ipsilateral to the corpus luteum. Control embryos were cultured with vehicle (0.1% ethanol). Cleavage and blastocyst rates were recorded at Day 2 and Days 6-7-8 post-insemination, respectively. At Days 7-8, blastocysts were evaluated for cell number after staining with Hoechst. Day-7 blastocysts were vitrified and evaluated for post-thawing survival for 72h (Live-Dead staining, confocal microscopy). Subgroups of fresh Day-7 blastocysts were individually evaluated for their lipid content by Intact Cell MALDI-TOF MS (ICM-MS) in positive ion and reflectron modes. The effect of hormonal treatments on embryo rates and cell number/MS data was evaluated by ANOVA and Kruskall Wallis tests, respectively. The effect on cryosurvival rates was evaluated by exact Fisher tests.
Exposure to steroids did not affect the proportions of embryos that cleaved on Day 2 (78-81%) and developed to the blastocyst stage on Days 6, 7 and 8 (25-31%, 31-36% and 30-37%, respectively; 6 replicates; n = 474 oocytes/group). The mean number of cells per blastocyst did not change between groups (97.8-118.2 cells, n = 10-24 embryos/group). However, P4 improved the rate of embryo survival at 24 h post-thawing compared with controls (95% vs. 65%, P = 0.04) while E2 improved embryo survival at 72 h post-thawing (55 vs. 20%, P = 0.02; n = 14-22 embryos/group). By ICM-MS, a total of 323 m/z within the 400-1000 mass range, corresponding mostly to phosphocholines and sphingomyelins, were detected. Among them, 119 masses were differentially abundant between groups (P < 0.01; fold-change >1.5 or <0.67; n = 11 embryos from 4 replicates/group). Exposure to P4/E2/CO induced the highest changes in embryo lipid composition, with 51 up-regulated and 30 down-regulated lipid species, followed by P4 (10 up- and 24 down-regulated), CO (6 up- and 27 down-regulated) and E2 (6 up- and 7 down-regulated lipid species). In conclusion, the exposure of bovine embryos to physiological concentrations of steroid hormones did not affect *in vitro* developmental rates but improved blastocyst quality through increased cryotolerance and altered lipid composition, in particular cell membrane phospholipids. Further studies are required to identify the lipids potentially linked to a better embryo cryosurvival and underlying mechanisms.
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Lipidomic profiling of the bovine oviductal fluid across the estrous cycle

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Keywords: bovine oviductal fluid, tubal, lipids.

Sperm capacitation, fertilization and early embryo development take place within the oviduct during the periovulatory period. Phospholipids (PL) are known to be taken up by the spermatozoa, influencing sperm capacitation. PL are also crucial for the embryo membrane fluidity, permeability and post-cryoconservation survival. However, data on the nature of PL present in the oviductal fluid (OF) and their regulation across the estrous cycle are scarce. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to monitor the abundance of phospholipids in the bovine OF according to the stage of the estrous cycle and the side relative to ovulation. Bovine oviducts were collected at a local slaughterhouse as previously described (*Lamy et al*, *Theriogenology 86:1409*, *2016*) and classified into 4 stages according to the ovarian and corpus luteum morphologies (n = 19 cows/stage): post-ovulatory (Post-ov), mid luteal (Mid-lut), late luteal (Late-lut) and pre-ovulatory (Pre-ov) stages of the estrous cycle. Follicular fluid was also collected from the Pre-ov follicles: animals with intra-follicular progesterone >160 ng/ml (cystic follicles) were excluded. Oviductal fluids were collected from contra- and ipsilateral oviducts by squeezing and stored in liquid N before analysis. Lipid spectral profiles of individual OF (0.5 µL, 5 technical replicates) were acquired in the m/z range of 400-1100 in positive reflector mode using an UltraFleXtreme MALDI-TOF MS (Bruker). Differential analysis between stages and sides were performed by Kruskal-Wallis (followed by Bonferroni post-test for pairwise comparisons) and Wilcoxon tests, respectively, on normalized intensities using the R software. Peaks were considered to be differentially abundant between 2 stages or sides when the p-value was <0.05[MSD1] and the ratio of normalized intensities >1.5 or <0.67. Principal component analyses (PCA) and hierarchical clustering (HC) were performed on most differential peaks ($P < 0.01$) using R. Lipids profiles were obtained for all OF samples: a total of 209 molecular species were characterized, including phosphatidylcholines, phosphatidylethanolamines and sphingomyelines. No significant differences were identified between ipsi- and contralateral OF at a given stage. However, in ipsilateral OF, 57 differential peaks were identified between stages, of which 52, 37 and 22 for Pre-ov vs. Mid-lut, Pre-ov vs. Late-lut and Pre-ov vs Post-ov comparisons, respectively. The PCA and HC clearly discriminated the Pre-ov stage from the three others.
In conclusion, a global lipidomic approach by MALDI-TOF profiling was applied to the OF for the first time. By this way, we demonstrated that PL in the bovine OF are highly regulated across the estrous cycle, in particular between Pre-ov and the lutal phase. The identification of the different PL species is ongoing. This work will provide new candidates potentially able to regulate sperm physiology and early embryo development.
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Short- and long-term effects of progesterone and prolactin during the second phase of IVM on metaphase-II chromosomes in bovine oocytes

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Keywords: progesterone, prolactin, M-II chromosomes.

Progesterone (P4) production by cumulus cells rises with oocyte maturation. We have previously shown that exogenous P4 and prolactin (PRL) exert similar inhibitory effects on abnormal changes of M-II chromosomes in bovine oocytes during the second step of two-step IVM (Lebedeva et al., Reprod Domest Anim, 52(S3):53, 2017). The goal of this work was to examine the role of the endogenous P4-related pathway on destructive modifications of M-II chromosomes and implementation of the PRL effect. Bovine cumulus-enclosed oocytes (CEOs) were matured for 12 h in control medium (CM; TCM + 10% fetal calf serum) containing 10 μg/ml FSH and 10 μg/ml LH at 38.5°C and 5% CO_2. The CEOs were further matured for 12 h in one of three IVM media: (1) CM, (2) CM containing 10 μM trilostane (TS, inhibitor of 3β-hydroxysteroid dehydrogenase), and (3) CM containing 1 μM mifepristone (MFP, antagonist of the genomic P4 receptor). The following additives to these three media were applied: no additives (Control), 50 ng/ml bovine PRL (Research Center for Endocrinology, Moscow, Russia), or 50 ng/ml P4. A part of CEOs matured for 12 h in all three media was cultured for additional 24 h in CM to test long-term effects during aging. At the end of culture, the state of oocyte chromosomes was evaluated by the Tarkowski's method. The content of P4 in culture media was determined by ELISA. Percentage data (n = 4-5, 81-106 oocytes per treatment) were arcsine transformed and analyzed by ANOVA. Except for PRL, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). After 24 h maturation, the rate of M-II oocytes was similar in all groups (80.1-92.6%). In the medium 1, both P4 and PRL reduced ($P < 0.05$) the frequency of M-II chromosome abnormalities (decondensation, adherence, clumping) from 28.4 ± 2.0% (Control) to 16.8 ± 2.7 and 15.2 ± 1.7%, respectively. In the media 2 and 3, the rates of M-II oocytes with abnormal chromosomes increased in all groups (at least $P < 0.05$); however, these rates were 1.5 times lower in the PRL-treated groups than in the respective control groups. After aging of control groups, a higher incidence of chromosome abnormalities ($P < 0.01$) was observed in CEOs exposed to TS (79.0 ± 2.0%) than MFP (67.3 ± 1.0%) or in untreated CEOs (66.6 ± 0.9%). The addition of P4 to IVM media 1 and 2 led to a 1.2-fold reduction ($P < 0.01$) in the rate of aged oocytes with abnormal chromosomes, while PRL exerted a similar long-term action only in the medium 2. Meanwhile, TS decreased ($P < 0.001$) the content of P4 in IVM media both in the absence of exogenous P4 (27 to 28 times) and in its presence (4 times), whereas PRL and MFP had no effect. This content remained low in aging media for CEOs matured with TS, although it rose 5 to 11 times in other groups. Thus, during the second phase of IVM, endogenous P4 can exert short- and long-term inhibitory effects on abnormal changes of M-II chromosomes, acting via genomic receptors, but it is not involved in the effect of PRL.

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The effect of short-term cytoskeletal inhibitor treatment on embryo metabolism and viability

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Keywords: metabolism, cytoskeleton, nuclear transfer technologies.

The short-term use of chemical inhibitors of the cytoskeleton in oocytes is necessary for a range of micromanipulation events, including mitochondrial replacement therapy (MRT) and somatic cell nuclear transfer (SCNT). These advanced methods have been shown to be effective; however the full safety of such techniques has yet to be demonstrated comprehensively. To begin to address this knowledge gap, we have measured the impact of short-term cytoskeletal inhibitor treatment on embryo development and metabolism. Abattoir-derived bovine oocytes were matured and fertilized under standard conditions. At the early pronuclear (ePN) stage, after 9 hours co-incubation with motile sperm, presumptive zygotes were exposed to one of cytochalasin B, latrunculin A, or nocodazole for 15 minutes. Mitochondrial response to treatment was assessed in real-time, and metabolic activity was measured in resultant embryos at cleavage and blastocyst stage. At these stages, treated embryos were transferred into 4µl individual culture droplets for 24 hours. Spent media was analysed using enzyme-linked fluorometric assays to measure glucose, lactate and pyruvate depletion/appearance and HPLC to measure the turnover of 18 key amino acids. In total, 36 embryos were assessed per group (three treatment groups and one control group) across 3 independent replicates at each developmental stage (acute, cleavage and blastocyst). Significance was tested to the level of $P < 0.05$ using one-way ANOVA with Tukey's post-hoc. The presence of cytoskeletal inhibitors at the ePN stage did not have an immediate impact on mitochondrial activity using a real-time assessment of response. Moreover, embryo development rate to cleavage (78.6 ± 6.4, 72.3 ± 4.8 and 70.1 ± 6.9 compared to 73.9 ± 6.9 in Cyt B, Lat A, Nocod and control respectively) and blastocyst stages (37.7 ± 3.7, 30.5 ± 2.8 and 29.8 ± 2.0 compared to 33.3 ± 3.0) was unchanged. Oxygen, glucose and pyruvate consumption were not significantly altered at either cleavage or blastocyst stage, however showed higher variance in embryos derived from zygotes exposed to cytoskeletal inhibitors. Altered turnover of arginine, glutamine, lysine, threonine and tyrosine was observed in at least one treatment group at later stages of development, and changes in trend in overall amino acid turnover were noted. These data indicate that short-term cytoskeletal treatment does not induce an immediate metabolic response and does not cause gross changes to mitochondrial function in pre-implantation stage embryos. A 15 minute exposure at the ePN stage does, however, induce subtle alterations in amino acid metabolism at both cleavage and blastocyst stages, indicating a legacy-effect on treated embryos. These data are critical to consider as MRT moves into the clinic and is also of relevance to SCNT. Overall, these findings support the need for further research to understand the impact of using cytoskeletal inhibitors during early embryonic development.

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The effect of chilling on the viability of *in vitro* produced bovine embyos

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Keywords: cattle, blastocyst, elongation.

Conventional freezing of *in vitro* produced bovine embryos has usually significant negative impact on subsequent embryo viability. Vitrification, on the other hand, does not allow direct embryo transfers. Since long distance shipment of fresh *in vitro* produced embryos is also not recommended without incubator-like shipment conditions, the aim of the present study was to evaluate the possibility to transport *in vitro* produced bovine embryos overnight as chilled aiming at direct embryo transfers on farms. Unless stated otherwise, all the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Slaughterhouse-derived oocytes were matured in TCM199 with glutamax-I (Gibco™; Invitrogen Corporation, Paisley, UK) supplemented with 0.25 mM Na-pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 ng/ml FSH (Puregon, Organon, Oss, Netherlands), 1 µg/ml β-estradiol (E-2257) and 10% heat inactivated FBS (Gibco™, New Zealand) at 38.5°C in maximal humidity in 5% CO_2 in air. Zygotes were cultured in G1/G2 media (Vitrolife, Göteborg, Sweden) supplemented with FAFBSA (4 mg/ml) and L-carnitine (1.5 mM) at 38.5°C in maximal humidity in 5% O_2, 5% CO_2 and 90% N_2. Transferable day-7 embryos were packed in straws in Hepes-buffered TCM199 supplemented with FAFBSA (1 mg/ml). After 24-h storage at 5°C embryos were either cultured for 72 h *in vitro* (until hatching) or transferred (10 embryos/recipient) into recipient heifers (until elongation). Following chilling, the *in vitro* hatching rates were lower for the morulae 18.2% (n = 22) than for the blastocyst stage embryos 68.5% (n = 89) ($P < 0.05$, Fisher exact test). Four day-7 recipient heifers were subjected to three separate embryo transfers each (a total of 120 blastocysts). Following the 12 non-surgical embryo collections on day 14 intact elongated embryos were recovered in seven collections (58.3%) yielding on average 4 embryos per collection. The average length of the elongated conceptuses was 9.95 mm (range 0.5-60 mm). In conclusion, using the presented conditions, *in vitro* produced bovine blastocysts are able to start elongation phase in recipient heifers after 24-h chilling period.
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Interferon tau exerts concentration dependent actions on bovine neutrophil gene dynamics favoring implantation

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Keywords: Interferon tau, neutrophils, implantation.

It is a proven fact that Interferon tau (IFNT) signals pregnancy recognition in ruminants. Apart from local actions on endometrium, it can reach systemic circulation and exert effects on various immune cells including neutrophils, thereby leading to immunomodulation via specific expression of interferon-stimulated genes (Meyerholz et al., Reprod Domest Anim 51(1):175-7, 2016). Though some studies have been performed to analyze the *in vivo* dynamics of neutrophils during bovine peri-implantation period (Shirasuna et al., Reproduction 150:217-225, 2015), there is no data available on exclusive effects of IFNT on neutrophils which is the basis of our study. The genes selected for the study were interferon-stimulated genes like Interferon-stimulated gene 15 (ISG15), 2'-5'-oligoadenylate synthetase 1 (OAS1), Interferon-induced GTP-binding protein MX1 and MX2, interferon-gamma-inducible protein 16 (IFI16), Interferon Induced Protein 44 (IFI44) along with other genes like Platelet-endothelial-cell adhesion molecule-1 (PECAM-1), L-selectin, Integrin alpha M and Progesterone-inducible molecule (PIBF) that determine many of the effector functions of neutrophils like motility, trafficking, phagocytosis etc. Healthy Sahiwal heifers (n = 10) aged 1.5-2 years were used for blood collection by jugular venipuncture [10 ml]. Neutrophils were isolated by established protocol [Manjari et al., Vet Immunol Immunopathol 173:44–49, 2016]. Cells were incubated with recombinant bovine Interferon-tau (MyBioSource Inc., San Diego, California, USA) in three different concentrations [1, 5 or 10 ng/ml] for 3 hrs at 37°C. At the end of the treatment, cells were retrieved from the wells for RNA isolation and gene expression study using real time PCR using specific primers for selected genes [Manjari et al., Vet Immunol Immunopathol 173:44–49, 2016; Biol Rhythms Res 49(2),329-333, 2018] and of CD31. The relative expression ratio of the target gene was calculated as per existing method (Livak and Thomas, Methods 25:402–408, 2001). All the data were analyzed by repeated measures one-way ANOVA for within group analysis followed by Fischer's multiple comparison test using SAS software, version 9.1 (SAS Institute inc., CARY, NC, USA). It was observed in the study that at lower concentrations of IFNT, ISG15, IFI16, PIBF and L-selectin were significantly up regulated, whereas at higher concentrations the same were significantly down regulated. Irrespective of concentration, MX genes, IFI44, and OAS1 were significantly up regulated and CD31, CD11b were significantly down regulated. The results show that at lower concentrations of IFNT, the neutrophil activity with respect to chemoattraction is stimulated whereas at higher concentrations the same is reduced. Hence, it can be concluded that IFNT exerts concentration dependent actions on neutrophil gene expression dynamics finely modulating its activity enabling the cells to exert temporal variation in their destined functions ultimately leading to successful implantation.
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Analyzing the effects of bovine Interferon tau and female sex steroids on neutrophil pro and anti inflammatory triggers to understand implantation

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Keywords: implantation, inflammation, neutrophils.

Bovine Interferon tau, a hormone of maternal recognition of pregnancy exerts both paracrine and endocrine effects on reproductive organs and also proven to have an effect on neutrophils (Shirasuna et al., Reproduction 150:217-225, 2015) which is partly understood till date. Though neutrophils are detrimental to the foreign invading antigen, they can also play a role in favoring implantation. Two gens that encode proteins important in the balance of inflammatory responses are IL-8 receptor (CXCR1) and Glucocorticoid receptor alpha (GRα) transcription factor. IL-8 binds CXCR1 and activates NF-κB associated proinflammatory pathways implantation whereas GRα can repress the activity of NF-κB thereby reducing inflammation. The differential roles of Interferon-tau [IFNT], Progesterone [P4] and Estradiol in triggering pro- and anti-inflammatory responses during peri-implantation period were assessed in cows. An ex vivo study was done by isolating neutrophils from the animals post artificial insemination (AI) at defined intervals (day 0, 4, 8, 12, 14, 16, 18, 21, 24, 30, 40) and analyzing the expression changes of GRα and CXCR1 genes. Isolation of neutrophils from the blood was performed as per the established protocol [Manjari et al., Vet Immunol Immunopathol 173:44–49, 2016]. An *in vitro* study was also done isolating neutrophils from prepubertal heifers and subjecting them to supplementation of IFNT (MyBioSource Inc., USA) [1, 5, 10 ng/ml], Progesterone and 17β-estradiol (Sigma Chemical Co., USA) at [10, 50, 100, 500, 1000 ng/ml], and [10, 50, 100, 500, 1000 ng/ml] respectively. Post supplementation, RNA was isolated from cells using Trizol method and was reverse transcribed to cDNA which was used for qPCR via LightCycler® 480 Instrument [Roche, Switzerland] using the SYBR Green kit [Thermo Scientific, USA] and specific primers for GRα, CXCR1, GAPDH, beta actin. Average values of the two housekeeping genes were used as reference for normalization of target gene for relative quantification. The relative expression ratio of the target gene was calculated as per established method (Livak and Thomas, Methods 25:402–408, 2001). Repeated measures one-way ANOVA was used for analysis of Data for within group analysis followed by Fischer's multiple comparison test using SAS software 9.1 (SAS Institute inc., USA). We observed that from day 14 post AI, there is a significant up-regulation of neutrophil GRα and CXCR1 in pregnant and non-pregnant cows respectively. The *in vitro* study showed that IFNT significantly up-regulates GRα whereas P4 significantly up-regulates CXCR1. Whereas estradiol significantly downregulates GRα but did not show any significant effects of CXCR1. The total leukocyte count and neutrophil count were also significantly higher in non-pregnant cows. We conclude that IFNT efficiently mediates neutrophil immunosuppression during peri-implantation period in cows by interacting with Interleukin 8 receptor and can also exert ligand independent actions on GRα.
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Role of RNA isoform expression in sex determination in mice

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Keywords: sex determination, splicing.

The majority of eukaryotic genes produce multiple transcriptional isoforms from the same locus. Thus, mRNA isoforms are molecules of different exon composition and length, which may code for different forms of the corresponding protein. They may be produced from different transcriptional starting sites, terminated at different polyadenylation sites, or as a consequence of alternative splicing (AS). As isoform changes may be masked by gene-level measurements, estimation of isoform expression provides a better resolution than gene expression to evaluate dynamic developmental processes. The genes that determine gonadal sex determination (GSD)in mice are known, but knowledge of the molecular pathways specifying GSD is still incomplete, in part because of these AS mechanisms. To identify differentially expressed isoforms (DEI), differentially expressed genes (DEG) and AS changes during GSD in mice, we performed a transcriptional analysis of RNA isoforms, genes and AS of XX and XY gonads during GSD at embryonic day 11 (E11) and early sex differentiation at day 12 (E12) using RNAseq. Two pairs of genital ridges (dissected from 2 different fetuses) were pooled per sample. Three samples were collected per sex. RNAseq libraries were prepared from male and female samples (n = 3 per sex) at E11 and E12. DEG was evaluated with DESeq and edgeR packages, and genes were considered differentially expressed when both tests returned a statistically significant result (cutoff: $P < 0.01$). Quantification of transcript abundance was performed with Salmon, and DEI analysis was carried out with edgeR package (cutoff: $P < 0.01$). AS events were evaluated with vast-tools software. Gene analysis identified 729 and 1691 DEGs between males and females at E11 and E12, respectively. Hundreds of these genes are related with GSD and early sex differentiation and could be good candidate genes for sex reversal; also, many of them appeared to be grouped in clusters on several chromosomes. Interestingly, increased expression at E11 in males was significantly enriched in RNA splicing and mRNA processing gene ontology (GO) terms. Isoforms analysis identified 705 and 1348 DEIs between males and females at E11 and E12, respectively. We found 14 genes at E11 and 19 genes at E12 with different isoforms expressed in males and females. Many DEI did not shown differences in the DEG analysis. In addition to the isoforms, 1167 differentially AS events were observed between females and males at E11. At E11 there was an enrichment in intron retention (IR) in females, and at E12 there was enrichment in IR and exon skipping in females. Eighty-five genes exhibited expression of different AS events in both males and females at E11, and 184 at E12. Some of these AS genes are transcription factors that could play an important role in GSD, like *Jarid2*a Jumonji family member essential for AS sex determination in reptiles. Our data indicate that RNA isoforms expression and splicing regulatory mechanisms constitute a common feature among sex determination in distant phyla, including mammals.
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A226E Embryology, developmental biology, and physiology of reproduction

The effect of vitamin c on the developmental competences and quality of pig blastocysts obtained after *in vitro* fertilization

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Keywords: pig, embryo, vitamin c.

Vitamin C is one of the antioxidants used in *in vitro* culture media for oocytes and embryos of animal species. It has been shown, that vitamin C has antioxidant properties and reduces the level of reactive oxygen species (ROS), which cause damage to structures in oocytes and embryos. The aim of the study was to determine the effect of vitamin C on developmental competences and the quality of pig embryos obtained after IVF and IVC. IVF embryos were obtained from *in vitro*-matured and *in vitro* fertilized oocytes. The putative zygotes were cultured in the NCSU-23 medium with the addition of 20 µg/mL of vitamin C (exp. group) or without it (control), at 39^{o}C and 5% CO2, 5% O2 and 90% N2 in the air up to the blastocyst stage. The IVF derived blastocysts from the exp. (n = 11) and control (n = 7) groups 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 filters: 520 nm (TUNEL) and 358-461 nm (DAPI). Statistical analysis was performed using the t-test and chi-square test. It was observed that the rate of embryos cleavage was slightly higher in the control group than in the exp. group (22.0% and 17.5%, respectively, no statistical differences). Simultaneously, the percentage of blastocysts was significantly higher in the exp. group than in the control group (51.3% and 16.7%, respectively $P < 0.01$). The largest percentage of blastocysts was obtained on day 6 of IVC supplementing with vitamin C (54.5%), while in the control group on day 7 (57.1%). It was observed also that the mean number of cell nuclei was slightly higher (41.4 ± 7.7) and the mean number of apoptotic nuclei was slightly lower (1.3 ± 1.2) in embryos cultured in the presence of vitamin C compared to embryos from the control group (37.0 ± 6.7 and 1.9 ± 1.8, respectively), but no differences were observed. The TUNEL index was 3.1% for the exp. group and 5.0% for the control group. The study showed that *in vitro* culture in the presence of vitamin C may improve production of pig blastocysts and has possibly no significant effect on their quality. It should be noted, these are preliminary results and it is planned to continue research on a larger number of pig embryos.
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A227E Embryology, developmental biology, and physiology of reproduction

Sperm storage in hen's reproductive tract: metabolic composition of the uterine fluid after artificial insemination

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Keywords: sperm storage, uterine fluid metabolite, ^{1}H-NMR.

Avian uterine fluid (UF) has been demonstrated to prolong sperm survival, maintain the fertility potential of the fowl sperm, and impact the filling rate of sperm storage tubules from utero-vaginal junction (UVJ) (Ahammad et al., Journal of Poultry Science, 50, 74-82, 2013). Two lines of hens previously selected on hatched chicks were used, one exhibiting a long (F+, 14-16 days) and the other one a short (F-, 4-6 days) duration of fertile period (Beaumont et al., British Poultry Science, 33, 649-661, 1992), as a major consequence of a good or a poor sperm storage ability, respectively (Brillard et al., Journal of Reproduction and Fertility, 114, 111-117, 1998). Therefore, UF analysis from F+ and F- hens represents a good approach to study molecules involved in UVJ sperm storage process. In this study, the comparison of UF metabolites from F+ (n = 5-7) and F- (n = 7-9) hens was investigated and quantified by high resolution proton nuclear magnetic resonance (^{1}H-NMR). Moreover, the effect of sperm storage on UF metabolic composition was investigated 24 hours, 1 week, 2 weeks or 3 weeks after artificial insemination (n = 5-9/ times). ^{1}H-NMR analysis was done on a Bruker DRX-500 spectrometer (Bruker SADIS, Wissembourg, France). Topspin 2.1 software and AMIX software package were used to process ^{1}H-NMR spectra prior to assign them using our in-house database and online databases, including HMDB (http://www.hmdb.ca) and the Chenomx NMR Suite 8.1 evualuation edition (Chenomx Inc, Edmonton, Canada). To identify discriminant metabolite between the two chicken lines and between the time-condition after insemination, Orthogonal Projections Latent Structures Discriminant Analysis (OPLS-DA) were performed using the SIMCA 13 software. Equality of means was tested using Welch's t-test for each discriminant metabolite ($P < 0.05$). To identify the most significantly affected metabolic pathways, the discriminant metabolite signature of each chicken line was analyzed by metabolite set enrichment analysis, implemented in Metaboanalyst 3.0 (htpp://www.metaboanalyst.ca). Eleven discriminant metabolites between the two lines were identified by OPLS-DA in the UF. There was an over-representation of inositol and galactose metabolism pathways in the UF of F- line ($P < 0.01$). On the other hand, metabolisms of steroid ($P < 0.001$), tryptophan ($P < 0.01$), arginine and proline ($P < 0.05$), as well as mitochondrial electon transport chain ($P < 0.05$), were over-represented in the UF of F+ line. While no metabolites were discriminant in F+ UF between before and after insemination, OPLS-DA revealed that ten metabolites were discriminant in F- UF. Among them, fumarate ($P < 0.05$) and myo-inositol ($P < 0.05$) metabolites were more concentrated after insemination than before, whatever the duration after insemination. In contrast, dimethylamine was less concentrated after insemination than before ($P < 0.05$). Our results indicate that metabolic composition of uterine fluid is associated with avian sperm storage duration in female genital tract and is related to sperm storage capacity.
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A228E Embryology, developmental biology, and physiology of reproduction

Local embryo effect on the transcriptomic response of the oviductal epithelial cells results from *in vivo* and *in vitro* approaches

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Keywords: bovine, oviduct-embryo interaction.

Based on previous data, the presence of a single 8-cell embryo does not alter the transcriptome of the cells of the oviduct, although this apparent lack of response might be due to a local effect at the precise position of the embryo which is missed if the whole oviduct is studied. Thus, we aimed to study the local embryo effect on the transcriptomic response of the epithelial cells of the oviduct *in vivo* and *in vitro*. For the *in vivo* experiment, 15 cross-bred beef heifers were synchronized, artificially inseminated and slaughtered on Day 2.5 after estrus. The oviducts from each animal were isolated, trimmed free of tissue and divided between ampulla and isthmus. The ipsilateral isthmus was then divided into smaller sections (2 cm). Each section was sequentially flushed until the embryo was located (n = 4; three at 2-cell stage and one at 8-cell stage), opened and scraped longitudinally to obtain the epithelial cells. Cells were snap-frozen in liquid nitrogen for gene expression analysis. The *in vitro* approach consisted of the co-culture of fifty *in vitro* produced embryos (2- to 4-cells) on a bovine oviductal epithelial cells (BOEC) monolayer. BOEC from the ampulla and isthmus of ipsilateral oviducts collected during the early luteal phase were mechanically harvested and separately cultured with TCM-199+10% FCS in 5% CO_2 in air at 38.5°C for 7 days until confluence. *In vitro* 2- to 4-cell embryos were produced in parallel. A day before co-culture, BOEC medium was replaced with SOF+5% FCS. The groups were: Ampullary BOEC co-cultured with (A+) and without (A -) embryos; isthmic BOEC with (I+) and without (I -) embryos. After 24h of co-culture, BOEC were recovered from each group and snap frozen for gene expression analysis (5 replicates). Ten transcripts previously reported to be differentially expressed between the isthmus of pregnant and cyclic heifers (*Maillo et al. Biol Reprod. 2015. 92: 144*) were analysed in BOEC recovered from both experiments: *STK32A*, *SLC26A3*, *KERA*, *QRFPR*, *MCTP1*, SOD3, *PRELP*, *VAT1L*, *SOCS3*, *CCL20*. Data were analysed using one-way ANOVA and t-test. The results from *in vivo* samples revealed that 6 out of 10 transcripts (*STK32A, SLC26A3, QRFPR, MCTP1, SOCS3* and *CCL20*) were different between the segment where the embryo was collected and other locations within the ipsilateral oviduct which suggested the presence of an embryo site-specific signal. Comparison between the ipsilateral embryo site with the contralateral site revealed only one transcript different (*VAT1L*). Regarding the *in vitro* BOEC co-culture, 3 out of 10 genes (*SLC26A3, KERA* and *QRFPR*) were not expressed. For the remainder of the genes analysed, no differences were detected. In conclusion, under our experimental conditions, *in vivo* the embryo elicits site-specific signals in the oviduct, while *in vitro* evidence for these signals were not observed neither by the presence of the embryo, nor by the spatial differences of the bovine oviduct.

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A229E Embryology, developmental biology, and physiology of reproduction

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

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Keywords: oviduct, hyperglycaemia, oviduct fluid.

The oviduct, or Fallopian tube, provides the environment for gamete activation, fertilisation and the early stages of embryo development. However, little is known of the composition of the oviduct fluid in health and disease. The aim of this study was to investigate the impact of a hyperglycaemic challenge on the physiology of oviduct epithelial cells and the composition of fluid that they create *in vitro*. We have used the bovine material as a model for determining the impact of metabolic diseases on human fertility. Primary bovine oviduct epithelial cells were cultured in DMEM-F12, at 39^{o}C, 5%CO_2 for 6 days. Cell identity was confirmed using confocal, optical and TEM microscopy. The cells were grown to confluence on a semi-permeable membrane. Barrier integrity was confirmed by measuring TransEpithelial Electrical Resistance (TEER) and fluorescein transport assays. Once confluence was achieved, the apical medium was discarded and cells were cultured in an air:liquid interface. Once confluence was confirmed, physiological (7.3mM) and hyperglycaemic (mild:8.5mM, severe:11mM) concentrations of glucose were added together with or without 20ng/ml of insulin to the basal compartment for either 24h (Experiment 1; *Acute*) or 7 days (Experiment 2; *Chronic*). The nutrient composition of apical secretions was analysed by enzyme linked assays and high-performance liquid chromatography. RNA was extracted from the cells for quantitative real-time PCR analysis of key genes related to oviduct physiology (*OVGP1*, *ERa*) and glucose transport (*INSR, SLC2A1, SLC2A3*). Insulin-only and glucose-free controls were included. Data were analysed using Kruskal-Wallis test with Dunn's post-hoc or two-way ANOVA where appropriate. Experiment 1 showed that an acute hyperglycaemic challenge in the basolateral compartment did not change the luminal concentrations of glucose, pyruvate or lactate, or the amino acid content of the cell secretions. By contrast, in experiment 2, 7-day basolateral exposure to hyperglycaemia in the absence of insulin reduced the volumes of oviduct-derived fluid (8.5mM+insulin and 11mM+insulin vs all treatments without insulin, $P < 0.05$), increased luminal concentrations of glucose (7.3mM+insulin and 8.5mM+insulin vs 11mM-insulin, $P < 0.05$) and modified the secretion of alanine, glycine, glutamine and arginine. Gene expression was not significantly modified in any of the genes tested, associated with oviduct epithelial cell function or glucose transport. Using an *in vitro* oviduct model, we have shown that exposure to hyperglycaemia in the presence or absence of insulin enriched oviduct secretions for glucose and modified the amino acid composition. Furthermore, insulin reduced fluid flow across the oviduct monolayer. These data suggest that the periconceptual environment can be modified in hyperglycaemia; such modifications may affect gamete and embryo physiology.

A230E Embryology, developmental biology, and physiology of reproduction

Effects of cumulus cells and prolactin on histone acetylation during the prolonged culture of matured bovine oocytes

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Keywords: oocyte aging, histone acetylation.

Aging of mammalian mature oocytes attained the metaphase-II stage heavily reduces their quality and developmental capacity. Therefore, the knowledge of physiological factors modulating the speed of oocyte aging is of great importance for successful assisted reproductive technologies. The goal of the present research was to study effects of cumulus cells (CCs) and pituitary hormone, prolactin (PRL), on the dynamics of age-associated epigenetic changes during the prolonged culture of bovine oocytes *in vitro*. Bovine cumulus-enclosed oocytes (CEOs) were cultured for 20 h in the following maturation medium: TCM 199 containing 10% fetal calf serum, 10 μg mL–1 porcine FSH, and 5 μg mL–1 ovine LH. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). After IVM, CEOs were transferred to the aging medium consisting of TCM-199 supplemented with 10% fetal calf serum and cultured for 0, 12 or 24 h, in the absence (Control) or presence of 50 ng mL–1 bovine PRL (Research Center for Endocrinology, Moscow, Russia). A portion of *in vitro*-matured oocytes were denuded of their CCs and cultured for 12 or 24 h in the control aging medium. At the end of maturation or prolonged culture, levels of acetylation of histone H4 at lysine 12 (acH4K12) and histone H3 at lysine 14 (acH3K14) in M-II oocytes were determined by immunostaining with specific antibodies (polyclonal rabbit anti-histone H4 lysine 12 and monoclonal rabbit anti-histone H3 lysine 14, both from Abcam, Cambridge, MA, USA). The fluorescence signal was evaluated using ZEN 2 Pro software (Carl Zeiss, Oberkochen, Gemany) and assigned to one of four grades (intense, moderate, weak and absent). Also, before analysis oocytes were counterstained with DAPI to visualize chromosomes. The data from 4 replicates (71-88 oocytes per treatment) were analyzed by ANOVA. In the control group of CEOs, a rise in the rate of oocytes with the intense fluorescence signal of acH4K12 occurred by 12 h of aging (64.1 ± 2.5% (12 h) v.52.7 ± 1.6% (0 h); $P < 0.01$) and persisted up to 24 h (86.5 ± 2.5%; $P < 0.001$). At the same time level of acH3K14 in aged oocytes increased only between 0 and 12 h of the prolonged culture (from 73.5 ± 1.8 to 92.0 ± 1; $P < 0.001$) and then this elevation ceased (93.5 ± 1.0 (24 h)). The addition of PRL to the aging medium or removal of CCs decreased levels of acH4K12 and acH3K14 in matured oocytes. In the PRL-treated groups, these effects were found after 24 h-aging, but in the groups of oocytes cultured in the absence of CCs, the rate of oocytes with the intense signal of acH4K12 and acH3K14 was lower than in the control group of CEOs throughout all culture periods (at least $P < 0.05$). Thus, elevated levels of H4K12 and H3K14 acetylation in matured oocytes during their prolonged culture suggest that these epigenetic changes may be caused by aging of ova. Furthermore, in bovine CEOs, CCs accelerate acH4K12 and acH3K14, whereas PRL decelerate these actions of CCs.

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A231E Embryology, developmental biology, and physiology of reproduction

Apoptosis resistance of bovine cumulus-oocyte complexes is modulated via progesterone-dependent pathways at the terminal step of *in vitro* maturation

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Keywords: progesterone, prolactin, oocyte apoptosis.

Cumulus-derived progesterone (P4) is an important pro-survival factor, with its production significantly increasing during bovine oocyte transition from M-I to M-II stage. The aim of the present research was to compare effects of endogenous and exogenous P4 during the terminal step of *in vitro* maturation on apoptosis resistance of bovine oocytes and cumulus cells. Bovine cumulus-oocyte complexes (COCs) were matured 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 and matured for 12 h in the following systems: (1) TCM 199 containing 10% FCS, (2) TCM 199 containing 10% FCS and 10 μM trilostane (TS, inhibitor of 3β-hydroxysteroid dehydrogenase), and (3) TCM 199 containing 10% FCS and 1 μM mifepristone (MFP, antagonist of the genomic P4 receptor). All these systems were supplemented with either 0 (Control) or 50 ng/ml of P4. After 24 h IVM, a portion of COCs was cultured for 24 h in TCM 199 containing 10% FCS to induce aging. Following culture, oocyte apoptosis was detected using the TUNEL kit (Roche, Indianapolis, USA); nuclei were stained with DAPI. Cumulus expression of pro-apoptotic marker Bax was assessed by immunocytochemical staining using Bax antibodies (Bio-Rad, Hercules, CA, USA). All data (n = 5, 83-100 oocytes per treatment) were arcsine transformed and analyzed by ANOVA. After 24 h IVM in System 1, the rate of M-II oocytes with apoptotic signs was lower in the P4-treated group than in Control (9.4 ± 1.1 vs. 17.1 ± 2.5%, $P < 0.05$, respectively). However, P4 did not affect this rate in the presence of TS or MFP. In the control group in Systems 1, the addition of MFP to the IVM medium resulted in a 3.2-fold decrease ($P < 0.05$) in the original apoptosis rate. Both TS and MFP enhanced 1.2 to 1.4 times ($P < 0.05$) Bax expression in cumulus cells surrounding matured oocytes, whereas P4 did not change it. In System 1 following 24 h aging, maturation of CEOs in the presence of P4 led to a reduction from 52.0 ± 5.1% (Control) to 28.3 ± 2.7% (P4) ($P < 0.05$) in the rate of apoptotic oocytes. In its turn, MFP diminished 3.2 times ($P < 0.001$) the apoptosis rate in the control group and did not affect this rate in the P4-treated group. The expression of Bax in aged cumulus cells increased 1.2 to 1.6 times ($P < 0.05$), but was not related to the presence of TS, MFP, or P4 in IVM media. Our findings indicate that, during the terminal step of IVM, exogenous P4 inhibits apoptotic processes in oocytes through non-genomic receptors only with the availability of cumulus-derived P4, whereas blocking of the genomic receptors, in itself, also leads to a decrease in oocyte apoptosis. At the same time the expression of pro-apoptotic Bax in cumulus cells is enhanced at inhibition of genomic P4 receptors and does not depend on exogenous P4.
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A232E Embryology, developmental biology, and physiology of reproduction

Rescue potential of supportive embryo culture conditions on bovine embryos derived from metabolically-compromised oocytes

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Metabolic disorders like obesity are linked to subfertility. The upregulated lipolysis leads to elevated saturated (stearic; SA, palmitic; PA) and unsaturated (oleic; OA) fatty acids (FAs), both in serum and in follicular fluid. While their ratio determines the severity of lipotoxicity, exposure to these FAs has a detrimental impact on oocyte quality. Insulin-transferrin-selenium (ITS, as a mitogenic and antioxidative support) is used in *in vitro* culture medium to maximize blastocyst yields. We hypothesize that supportive culture media (containing e.g. ITS) can minimize cellular stress levels and rescue development and quality of embryos derived from metabolically-compromised oocytes. In this study, bovine oocytes were exposed to different ratios of PA, SA and OA; 1) pathophysiological concentrations: 150, 75 and 200 µM respectively (HI COMBI), 2) only high PA: 150, 28 and 21 µM respectively (HI PA), compared to 3) physiological basal concentrations: 23, 28 and 21 µM respectively as a control (BASAL). Presumptive zygotes were cultured in SOF medium with or without ITS. Cleavage rates were recorded 48h post insemination (p.i.) and blastocyst rates at day 7 (D7) and 8 (D8) p.i. (n = 905 oocytes, 3 repeats). D8 blastocysts were evaluated for apoptotic cell indexes (n = 227) by caspase-3 immunostaining or snap frozen for mRNA expression of genes involved in ER unfolded protein responses (UPR) (*Atf4, Atf6*), oxidative stress (*SOD2, GPx, CAT*) and mitochondrial UPR (*HSPE1, HSPD1*) (n = 356). Categorical and numerical data were analysed using binary logistic regression and ANOVA, respectively, and were Bonferroni-corrected for multiple testing. In the absence of ITS during culture, HI PA exposure during maturation significantly reduced cleavage (64.2% *vs.* 78.3%) and D7 blastocyst rates (12.4% *vs.* 24.3%) and tended to reduce D8 blastocyst rates (22.0% *vs.* 32.5% P = 0.098) compared to BASAL. Maturation in HI COMBI had no effect on development. However, surviving blastocysts derived from HI PA- and HI COMBI-treated oocytes showed a significant increase in apoptosis. In the presence of ITS during culture, maturation in HI PA or HI COMBI had no significant effect on developmental competence whereas apoptosis was not alleviated by ITS. Within the HI PA group, ITS supplementation rescued embryo cleavage rate (by 15.1%, $P < 0.05$), proportion of ≥4-cell embryos (by 13.9%, $P < 0.05$) and tended to increase D7 blastocyst rate (by 8.2%, P = 0.076) compared to the HI PA-treated group cultured without ITS. In the absence of ITS during culture, HSPD1 expression of D8 blastocysts from PA-treated oocytes was significantly increased compared to BASAL-treated oocytes ($P < 0.05$), an effect that was normalised by ITS. Within the HI PA-treated group, ITS tended to decrease HSPD1 expression (P = 0.069). Other genes were not affected. We conclude that ITS supplementation during embryo culture enhances development and alleviates mitochondrial stress (at mRNA level) of embryos derived from metabolically compromised oocytes (HI PA). However, produced embryos still showed higher apoptosis, indicating inferior quality.

A233E Embryology, developmental biology, and physiology of reproduction

Oviductal cells express Stearoyl-CoA desaturase that can protect the embryo against saturated fatty acids

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Keywords: NEFA, oviduct, embryo.

Metabolic stress conditions, characterized by elevated free fatty acid (NEFA) levels are associated with reduced fertility in mammals. Particulary saturated NEFAs have a negative impact on the developmental competence of the oocyte (Leroy et al., Reproduction; 130: 485-495, 2005), while mono-unsaturated NEFAs, present at relatively high levels in follicular fluid (Aardema et al., Biol Reprod; 88: 164, 2013), are able to protect against lipotoxic events induced by saturated NEFA (Aardema et al., Biol Reprod; 85: 62-69, 2011). Cumulus cells protect the oocyte against lipotoxicity by Stearoyl-CoA desaturase I (SCD-I) activity that converts saturated stearic acid (SA) into mono-unsaturated oleic acid (OA) (Aardema et al., Biol Reprod; 96:982-992, 2017). The oocyte appears to mature in a 'protected environment', which may be in contrast to the metabolic condition in the oviduct. The current study investigates the impact of physiologic NEFA levels on embryos and whether oviduct epithelial cells (OECs) express SCD-I. Cumulus-oocyte-complexes (COCs) originating from 2-8 mm follicles of bovine slaughterhouse ovaries were collected, *in vitro* matured (day -1) and fertilized (day 0) according to our standard protocol (Aardema et al., Biol Reprod; 85: 62-69, 2011). From day 1 embryos were exposed to CONTROL, or to BASAL NEFA (100 µM OA + 80 µM palmitic acid (PA) + 70 µM SA; the 3 dominating NEFAs in follicular fluid) or HIGH NEFA levels (200 µM OA + 150 µM PA + 100 µM SA) during the complete embryo culture period. Fatty acids (10 mM; NEFA) were complexed to BSA (FA:BSA ratio of 5:1). At day 8 of culture, blastocyst rate was scored. OECs were collected from infundibulum, ampulla and isthmus, at the pre-and post-ovulatory, early-and late-luteal phase of the reproductive cycle for quantitative RT-PCR analysis. RNA was extracted and reverse transcription was performed on total RNA, *SCD-I* mRNA expression levels were normalized by the geometric mean of reference genes *GAPDH* and *ACTB*. Statistical analysis was performed with SPSS 24.0, by general linear model. Exposure to NEFA dring embryo culture resulted in a significantly reduced blastocyst rate versus the control condition at day 8 of culture (19.7 ± 2.7%), for the BASAL NEFA (7.2 ± 4.5%, $P = 0.021$) and HIGH NEFA condition (3.7 ± 3.6%, $P = 0.020$; n = ~340 COCs, 3 runs per group). The *SCD-I* expression in the isthmus region was higher during the pre- versus the post-ovulatory and early-luteal phase ($P < 0.05$). Interestingly, *SCD-I* mRNA was expressed in each region of the oviduct during all phases of the reproductive cycle. These data indicate that embryos are very prone to NEFA and are threatened by metabolic stress. In particular since total NEFA levels in oviductal fluid resemble the levels in blood (Jordaens et al., Reprod Biol; 17:281-284, 2017). At this point, the NEFA composition in the oviduct is largely unknown. A major question, which is currently investigated, is whether the OECs that express SCD-I are capable of influencing the NEFA composition in the oviduct in the benefit of the embryo.

A234E Embryology, developmental biology, and physiology of reproduction

Enhancement of the developmental capacity of metabolically compromised bovine oocytes and embryos by water soluble vitamin E (TROLOX) depends on the timing of the treatment

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Maternal metabolic disorders are associated with elevated concentrations of free fatty acids (FFA) in serum, follicular- and oviductal fluid. Previous studies have shown that pathophysiological FFA concentrations, and in particular the saturated palmitic acid (PA), jeopardize bovine oocyte and embryo developmental competence *in vitro*. Moreover, gene expression and proteomic analysis of FFA exposed bovine oocytes point towards oxidative stress related pathways. As such, antioxidants may be a key factor in improving oocyte and embryo developmental competence. We investigated if the use of TROLOX, a water soluble vitamin E analogue and antioxidant, during IVM or IVC could enhance developmental competence of PA-exposed oocytes and embryos *in vitro*. Hereto, 1279 bovine oocytes were routinely matured, fertilized and cultured until day 8 in 2 different experiments (6 repeats each). In Exp.1, oocytes were exposed to pathophysiological follicular PA concentrations (150µM), after which the zygotes were cultured under solvent control (ethanol, PA-SC) or TROLOX (100µM, PA-TROLOX) conditions. In Exp.2, oocytes were matured under SC or TROLOX (100µM) conditions, then exposed to pathophysiological oviductal PA concentrations (230µM) during culture (SC-PA, TROLOX-PA). In each experiment a solvent control was included (SC-SC). Cleavage (48h post insemination, pi), blastocyst rates (D8 pi), the rates of D8 blastocysts/cleaved zygotes and the rates of D8 expanded and hatched blastocysts/total blastocysts were calculated. Developmental competence data were compared using a binary logistic regression model and Bonferroni post-hoc test (IBM SPSS Statistics 24). In Exp.1, cleavage of PA-SC (71%) was not significantly different from SC-SC (79%, $P = 0.133$). D8 blastocyst rates of PA-SC (22%) tended to be lower compared with SC-SC (32%; $P = 0.064$). Compared to PA-SC, we showed that TROLOX during IVC was not able to neutralize the PA insult during IVM (PA-TROLOX, 23%; $P > 0.100$). The rates of total D8 blastocysts/cleaved zygotes and D8 expanded and hatched blastocysts/total blastocysts were not significantly different. In Exp.2, cleavage, D8 blastocyst rates and D8 blastocysts on total cleaved zygotes of SC-PA (59%, 9%, 14%, respectively) were significantly reduced compared with SC-SC (79%, 32%, 39%, respectively; $P < 0.0001$). Cleavage and D8 blastocysts/cleaved zygotes of TROLOX-PA (68% and 24%, respectively) tended to be improved compared with SC-PA ($P < 0.1$). Moreover, the addition of TROLOX during IVM could significantly increase D8 blastocyst rates (17%) of PA-exposed embryos ($P = 0.022$), but not to control levels (32%). TROLOX during IVM significantly improved blastocyst development into expanded and hatched blastocysts when embryos were exposed to PA (SC-PA, 49% vs. TROLOX-PA, 68%; $P = 0.025$) to levels similar to controls (SC-SC, 63%). In conclusion, the antioxidant TROLOX can protect oocytes from metabolic stress insults after fertilization, but metabolically compromised oocytes cannot be rescued by the addition of TROLOX during embryo culture.

A235E Embryology, developmental biology, and physiology of reproduction

Elevated non-esterified fatty acid concentrations during *in vitro* maturation affect the transcriptome profile of day 14 bovine embryos 7 days after transfer

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Keywords: NEFA, transcriptome, embryo transfer.

We showed earlier that exposure to elevated non-esterified fatty acid (NEFA) concentrations during *in vitro* oocyte maturation (IVM) affects post-hatching development of day (D) 14 bovine embryos (Desmet *et al.*, Anim. Reprod. 14,p947,2017). Lipotoxic conditions during IVM influence DNA methylation in the D7 embryo and may thus affect its transcriptome during later development. Therefore the aim was to analyse the transcriptome profile of D14 embryos to investigate which mechanisms mediate carryover effects of adverse maturation condition on post-hatching development. Bovine oocytes were matured for 24h under 2 conditions: 1) basal physiological NEFA conditions (BAS; 28µM stearic acid (SA), 21µM oleic acid (OA), 23µM palmitic acid (PA)); and 2) high PA concentration (most predominant in follicular fluid during negative energy balance) (HPA; 150µM PA, 28µM SA, 21µM OA). After fertilization, zygotes were cultured in SOF with serum. 8 blastocysts (normal and expanded, equally distributed per treatment and per replicate) were transferred to healthy non-lactating Holstein Friesian cows at D7 (n = 8, 5 replicates). Each cow was used once for each treatment in a cross-over design. After transcervical recovery, D14 concepti (n = 45) were dissected into embryonic disc (ED) and extra-embryonic tissue (EXT). ED (n = 11BAS/7PA) and EXT (n = 13BAS/8PA) were subjected to RNA sequencing (without RNA amplification). Differential expression was established in a DESeq2 model based on Negative Binomial distribution. Samples were divided by sample type for further analysis. A false discovery rate (FDR) of 10% was used as cut-off for differentially expressed genes (DEG) and *P*-values were Benjamini-Hochberg corrected. Ingenuity Pathway Analysis (IPA) and Gene Set Enrichment Analysis (GSEA) were performed. Recovery rate at D14 was not significantly different between treatments. Within ED and EXT datasets, only 14 and 0 DEGs were detected in HPA embryos compared to BAS embryos, respectively. However, when comparing concepti of similar morphological class (spherical/ovoid/tubular) and sex, higher numbers of DEGs could be detected (e.g. in ED dataset up to 6 times more DEGs). Overall, more DEGs were observed in ED compared to EXT at each morphological stage (except male tubular embryos). IPA and GSEA showed that affected pathways were related to cell growth and adhesion, metabolism, endoplasmic reticulum stress, mitochondrial respiratory chain complex and epigenetic mechanisms. To conclude, elevated PA exposure during IVM has carryover effects on the transcriptome profile of D14 concepti although only good quality D7 embryos with the same morphology have been transferred. D14 transcriptome patterns were dependant on morphology (elongation stage) and cell type (ED versus EXT) but common pathways affected were related to cellular development, metabolism and epigenetics. This suggests that metabolic stress during oocyte maturation may have long-lasting effects on embryo development that may lead to reduced fertility in high-yielding dairy cows.

A236E Embryology, developmental biology, and physiology of reproduction

The Effects of undernutrition and supplementation with cactus silage in Boujaâd ewes on offspring growth performances

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Keywords: dietary restriction, cactus silage, physiological stage.

In Moroccan agriculture, sheep farming is extremely diversified but it is facing many constraints like the extensive mode of its management and the effects of climate change. The later makes food resources getting more scarced, thing that can affect the survival of the embryo and fetus development. In this context the cactus plant is currently gaining an interest in several countries to feed animals as it ensur a food supply in the case of a critical drought situation (Arba et al., *Agri.Mar,* 2009, 215-222). Thus, the aim of this work was to study the effects of nutrient restriction and total substitution of concentrate by cactus silage in Boujaâd ewes during late pregnancy and early lactation on neonatal offspring performance. The experiments were carried out at INRA-CRRA-Settat- MAROC. Thirty-three Boujaâd ewes at similar ages (3 months of gestation) and with an average weight of 50.83 ± 1.49 kg were used. The experiment started at 3 month ewe's gestation until 1 month lactation. Ewes were randomly assigned to three groups, a control group CG (n = 11), a dietary restricted group RG (n = 11) (deficit ration in energy and proteins) and a group cactus-based diet CBG (n = 11). The CG and CBG groups received the same energy amount. The food rations, adopted in this work were randomly selected in a way to simulate the under nutrition in small livestock producers during drought periods in Morocco. The body weight was measured monthly for all ewes. While, the lamb's body weight was measured at birth and one month later and lamb mortality and the rate of abortion were assessed. Statistical analyzes were performed using the JMP SAS 11.0.0 program and lamb mortality and the rate of abortion data were assessed by X2 analysis of contingency tables. This study showed significant effect ($P < 0.05$) on all parameters. The ewe body weight in late pregnancy and early lactation was higher in CG (53.36 ± 1.25 kg and 49.21 ± 2.09 kg respectively) compared to the RG (49.71 ± 2.08 kg and 46.02 ± 0.98 kg respectively) and CBG (49.18 ± 2.24 kg and 45.92 ± 0.98 kg respectively). The rate of abortion and mortality after birth were higher in RG (27.27% and 36.36% respectively) compared to the CG (7% and 8.2% respectively) and CBG (4% and 5% respectively). The weight of lambs at birth was significantly affected by the diet adopted, the weight was higher in CBG (3.4 ± 0.51 kg) and CG (3.12 ± 0.34 kg) compared to RG (2.4 ± 0.12 kg). Similarly, weight of lambs one month later was higher in CBG (18.1 ± 0.91 kg) and lower in the RG (6.98 ± 2.15 kg), while in CG the weight was intermediary (11.16 ± 0.28 kg). It is concluded that ewe undernutrition in late gestation and early lactation affect negatively lamb growth performances and mortality rate. However, the use of cactus silage as a feed for ewes can be used as an alternative to concentrate especially in arid area with limited food resources.

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Piglets obtained by transfer of embryos received after *in vitro* fertilization of oocytes matured with thymosin: a preliminary study

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Keywords: pig, oocyte, thymosin.

One of the most important issues of *in vitro* production of pig embryos is the quality of oocytes. Thymosin (TH) is a 28- peptide hormone, released by the Thymus. TH accelerates the healing of dermal burn wounds, injured cells or tissues as well as regenerates skin and stimulates hair regrowth (Tseng et al., 2002, Kim and Kwon, 2014). Until now the regeneration functions were examined only on somatic cells. In our recent study we showed that maturation of pig oocytes in a medium supplemented with thymosin increased the number of matured oocytes with lower morphological quality and improved the quality of *in vitro* obtained pig blastocysts (Gajda et al., 2015). The purpose of this preliminary study was to investigate the effect of maturation of oocytes with or without thymosin on *in vivo* survival of pig embryos obtained after *in vitro* fertilization. Cumulus-oocyte complexes (COCs) were obtained from ovaries collected from slaughtered gilts. COCs were selected based on cytoplasm morphology and cumulus cell layers appearance and cultured for 42–44 h in a modified TCM-199 medium supplemented with 1 mg/ml of synthetic TH (LipoPharm.pl) (experimental group) or without TH (control group). After maturation, oocytes from the experimental and control groups were assessed and fertilized *in vitro*. Presumptive zygotes were cultured in the NSCU-23 medium for 72 h at 39^{o} C, in an atmosphere of 5% CO_2 and 5% O_2 in humidified air. Embryos were transferred surgically, under general anesthesia, into a single oviduct of synchronized 6 month-old recipients gilts. Each of the two gilts from the experimental group and one from the control group received fifty embryos. On days 28-30 and 40, the diagnosis of pregnancy was performed by ultrasonography. The pregnant recipients were monitored until parturition, and the total number of piglets born within individual litters was determined. After transfer of embryos from the experimental group (50 to each gilt) into 2 recipients and 50 from the control group into 1 recipient, both gilts that received embryos obtained after *in vitro* fertilization of oocytes matured with TH became pregnant and delivered a total of 16 live piglets. Pregnancy was not achieved after transfer of embryos from the control group. In conclusion, in our preliminary study we suggest that the maturation of pig oocytes with thymosin supports the *in vivo* survival of IVF embryos. This study permitted us to set up the IVP-embryo transfer successfully in our lab allowing the birth of the first piglets obtained after IVP and ET in Poland. Further experiments are ongoing to validate the results of this study.

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A238E Embryology, developmental biology, and physiology of reproduction

PredOSEgenesis: A two-layer classifier for identifying oogenesis, spermatogenesis and embryogenesis-related proteins

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Keywords: SVM, protein, fertility.

Successful spermatogenesis and oogenesis are the two genetically independent processes preceding embryo development. Therefore, further studies are required to discover more proteins associated with the development of germ cells and embryogenesis in order to shed more light on the processes. Here, we extended our previous study (Rahimi, M., Bakhtiarizadeh, M. R. & Mohammadi-Sangcheshmeh, A. OOgenesis_Pred: A sequence-based method for predicting oogenesis proteins by six different modes of Chou's pseudo amino acid composition. J Theor Biol 414, 128-136, doi:10.1016/j.jtbi.2016.11.028 (2017)) and offered a new algorithm to predict not only the proteins are involved in oogenesis, but also those implementing spermatogenesis and embryogenesis processes. First we extracted 345, 641, and 831 proteins through searching the UniProtKB database with gene ontology terms for “oogenesis”, “spermatogenesis” and “embryogenesis”, respectively. Then we developed a new method based on the support vector machine (SVM) and informative protein physicochemical properties (1920 different features including amino acid composition, autocorrelation, Quasi sequence-order, two types of pseudo-amino acid compositions and etc.) for predicting new fertility-related proteins and their classes (oogenesis, spermatogenesis and embryogenesis). Moreover, we employed a feature selection approach by using 10 different feature weighting methods on general datasets (combination of oogenesis, spermatogenesis and embryogenesis) to identify the more important protein features for fertility/non-fertility-related proteins. Our model achieved 80.79%, 80.54% and 79.74% prediction accuracy by five-fold cross validation test and 82.03%, 80.15% and 77.16% prediction accuracy using the independent test for datasets with 50% identity for Oogenesis, spermatogenesis and embryogenesis respectively. Furthermore, our results of feature selection revealed that Isoleucine and Serine frequency, Dipeptide Composition, Quasi sequence-order, composition, distribution and conjoint triad are important features for fertility-related proteins prediction. Interestingly, we found that the role importance of serine and isoleucine in fertility-related proteins and related biological process are highlighted. Isoleucine is believed to be related to male fertility through its synthetic and metabolic activities. For instance, mutation of encoding gene of ubiquitin-specific protease 26 (responsible for a valine to isoleucine change) has been reported to cause male infertility and adversely affect the testicular function. Cytochrome P4501A1 participates in isoleucine–valine exchange; mutation of its heme-binding region is also associated with infertile men. The importance of isoleucine in sex-determining region Y (SRY) protein has been highlighted, specifically the orientation of isoleucine side chain in DNA minor groove. The key role of the glycine and serine-rich sequences in oogenesis and folliculogenesis processes is highlighted by explaining their relationship to bone morphogenetic protein (BMP) family. Finally, we developed a two-layer classifier software, named as “PrESOgenesis”.

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Characterization of sperm-oviduct extracellular vesicles interactions at various stages of the bovine estrous cycle and their effects on sperm physiology

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Keywords: extracellular vesicles, sperm-oviductal interactions.

Extracellular vesicles (EV), recognized as an important mechanism of cell-to-cell communication, have been identified in the oviductal fluid (OF) and have been proposed as key modulators of sperm-oviduct interactions. Hence, the aims of this study were: 1) to characterize sperm-oviduct EV interactions across the estrous cycle and 2) to identify the effects of these interactions on sperm viability and motility. Oviducts with their attached ovaries were collected from bovine reproductive tracts at a local slaughterhouse and classified into 3 stages of the cycle: Pre-Ovulatory (PE), Post-Ovulatory (PO) and Luteal Phase (LP). After OF collection, EV were isolated by ultracentrifugation and resuspended in 50 µL PBS (pool 300 µl from 2-3 cows). A pool of frozen-thawed washed spermatozoa (spz) from 2 bulls (Holstein and Normand; 85% viability and 60% motility after Percoll) was used. To analyze sperm-EV interactions and survival, EV were labeled with a green fluorescent dye (PKH67) and co-incubated (at 3.3% (v/v)) with spz for 2, 8 or 16 h at 37ºC, 5% CO_2in IVF media (Tyrode medium), then stained with Hoechst 33342 and Ethidium Homodimer (to visualize dead spz) and evaluated by confocal microscopy (~900 spz counted). Sperm motility was assessed after 2, 8 or 24 h of co-incubation with and without (Control) EV (at 1 or 10% (v/v)) by CASA. Each experiment was done in triplicate. The effects of the stage on interactions and of interactions on sperm viability were analyzed by chi-squared statistical test. The effect of interactions on sperm motility were analyzed by Kruskal-Wallis test. The proportion of spz interacting with EV (EV-spz) increased over time, ranging from 1-2% of EV-spz after 2h of co-incubation to 4-10% after 8h and 62-80% after 16 h. Overall, a significantly higher proportion of spz were bound to LP-EV compared to PE- and PO-EV (8h: 9% vs 5 and 4% and 16h: 76% vs 66 and 63%, respectively; $P < 0.01$). Besides, five different patterns of sperm-EV interactions were observed with a marked effect of the stage of the cycle. After 16h of co-incubation, PE- and PO-EV were mainly detected over the sperm head in comparison to LP-EV (70 and 54% vs 29% of EV-spz, respectively; $P < 0.01$) whereas LP-EV were distributed over the head and intermediate piece compared to PE- and PO-EV (58% vs 41 and 28% of EV-spz, respectively; $P < 0.01$). LP-, PE and PO-EV had a detrimental effect on sperm viability compared to controls at 8h (48- 52% vs 61%) and 16h (27-28% vs 35%; $P < 0.01$). Furthermore, PE-EV (10%) decreased significantly the % of motile & progressive spz after 2h incubation (10.3% vs 31,1% and 4.0% vs 19.3%; $P < 0.05$), and PO-EV (10%) the % of progressive spz after 8h incubation (2.6% vs 5.4%; $P < 0.05$). In conclusion, we showed that oviductal EV interacted with cryopreserved bovine spz in a stage-specific manner. Long co-incubation times with EV impaired sperm viability and motility. These results pave the way for further research on sperm-EV interactions in the maternal tract.
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A240E Embryology, developmental biology, and physiology of reproduction

Health status in the Latvian native breed of Latvian Brown dairy cows that are intended for multiple ovulation and embryo transfer

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Keywords: extinct cow breed, preservation.

The Latvian Brown (LB) dairy cows are native cattle breed endemic in Latvia. It is possible to save LB cow breed by using multiple ovulation (MO) and embryo transfer (ET) due to the ERAF project No. 1.1.1.1/16/A/025, *BioReproLV*. In Latvia MOET in cows has been restarted after 35 years of interruption. Many of the LB gene fond (GF) animals are at advanced age, they live in small farms without a calculated feeding ration, and many of these small private farm owners do not have appropriate education relevant to the cow physiology beside their own experience. The aim of the present study was to find out the health status in genetically valuable LB GF cows intended for MOET. Clinical examination, blood morphological (leucocytes, erythrocytes, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, platelets) and biochemical analysis (alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), gamma glutamyltransferase (GGT), alkaline phosphatase (AP), urea, creatinine (CREA), total protein (TP), albumin (Alb), sodium (Na), potassium (K), calcium (Ca), phosphorus (P), chloride (Cl), cholesterol (CHOL), triglycerides (TG), as well as glucose (Glc) and ß-hydroxybutyric acid (BHB) by using express test *FreeStyle Optimum Neo H*) were performed in 12 animals. Data are expressed as the mean ± SD and one sample t-test and independent samples t-test were performed for statistical analysis considering the significance level of P = 0.05. The donor cows were 5.3 ± 2.66 years old (max. 9). Body score condition = 2.0 points was in 3 cows (25%) and = 3.5 was in 4 cows (33%). No signs of illness were established by general clinical and reproductive tract examinations in 9 cows (75%), but 3 cows (25%) were rejected because of ovarian cysts, pyometra and subclinical ketosis. Blood morphological parameters were in the reference ranges in all cows. ALAT (40.3 ± 6.60 u/L) was increased in 4 (33%), GGT (39.5 ± 6.72 u/L) was increased in 7 (58.3%) and BHB (1.7 ± 0.25 mmol/L) was increased in 3 cows (25%), (P = 0.05). Urea (1.6 ± 0.86 mmol/L) was decreased (P = 0.05) in 4 cows (33%). These results indicate impaired liver health and functionality due to inaccuracies in nutrition. Other biological parameters as ASAT (84.4 ± 17.86 u/L), AP (60.0 ± 83.16 u/L), CREA (72.9 ± 27.81 µmol/L), TP(73.6 ± 6.39 g/L), Alb (32.5 ± 5.27 g/L), Na (142.3 ± 2.3 mmol/L), K (5.1 ± 0.48 mmol/L), Ca (2.5 ± 0.63 mmol/L), P (2.1 ± 0.33 mmol/L), Cl (98.7 ± 1.92 mmol/L), CHOL (4.5 ± 1.69 mmol/L), TG (0.2 ± 0.06 mmol/L), Glc (2.9 ± 0.72) were in the reference ranges in all cows. MO using pregnant mare serum gonadotropin and double artificial insemination were carried out for 7 cows till now and only 2 embryos were obtained. In conclusion, because of the inapropriate LB GF health status, it is necessary to conduct blood morphological and biochemical analyses in all cows intended for MOET. The express-tests (Glc, BHB) are the first signals of inaccuracies regarding cow metabolism, and blood biochemical analyses have proved it.