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Fertility effects of performing ovum pick up at young age

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Keywords: fertility, flushing AI index, IVP, OPU.

To shorten the generation interval, and to increase the genetic progress, CRV started to perform Ovum Pick Up (OPU) at 9 months instead of 12 months of age (Reproduction Fertility and Development 12/2014; 27;:209). We demonstrated that animals that had their (first) estrus before the first OPU produced significantly more embryos than animals that did not show estrus before OPU. It is however not known however what the effect is of performing OPU on such young animals on their fertility (i.e. flushing results and AI index).The aim of this study is to check the fertility of animals that have been used for OPU at young age. To investigate this we compared the flushing results and AI index of these animals. Embryos were produced by OPU-IVP (once every week during a period of 4-9 weeks), followed by flushing (two times) and insemination (AI) to make the animals pregnant. We used 3 groups of animals, (1) 12 young animals (9-10 months) that had their first estrus before the OPU, (2) 24 young animals that did not had their first estrus before the OPU and a (3) control group of 16 older (12-14 month) animals. The flushing results from young animals that had their first estrus before OPU (Group 1) were comparable with those of the control group (both 6.5 embryos per flush). However, flushing results from young animals that did not had their first estrus before OPU (group 2) were clearly lower and had only 4.1 embryo per flush.Interestingly, the insemination results (AI index) showed the same tendency, e.g. animals that had their first estrus before needed 2.1 semen straws to get pregnant, while animals that did not had their estrus before OPU needed 2.6 straws. It is therefore concluded that in young animals that showed estrus before the first OPU no difference in flushing results and AI index fertility results later in life were observed as compared to older animals. However, when no estrus was observed before the fist OPU, fertility results were lower. It is not known if this is due to the OPU at young age or that these are less fertile animals having estrus at a later stage.

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The use of neutral red as a viability indicator hampers *in vitro* development of semi-nude bovine oocytes to the blastocyst stage

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Keywords: Neutral Red, oocyte viability, semi-nude oocytes.

Women suffering from premature ovarian failure due to cancer treatment can appeal to oocyte vitrification to preserve their fertility. An important factor to increase the effectiveness of the procedure is viability assessment of the cryopreserved oocytes after warming. To date, survival is predominantly assessed on the basis of morphological criteria by conventional light microscopy, a subjective assessment method that depends largely on the expertise of the observer. Therefore, there is a great need for an objective method to assess viability in a fast and non-invasive way. Oocytes can be cryopreserved at the immature or mature stage. After choosing to use mature oocytes, based on literature, this experiment examined whether the relative non-toxic stain Neutral Red (NR) can be used as an oocyte viability marker without affecting subsequent development to blastocysts. NR is taken up by lysosomes of metabolically active cells. Briefly, immature cumulus-oocyte-complexes (COCs) were subjected to routine *in vitro* maturation (IVM) for 21 or 24 hours, whereupon the 270 mature COCs were divided into 3 groups (2 replicates). A control group with an intact cumulus oophorus (24h IVM; LAB CTRL) and 2 groups of COCs with only the corona radiata (21h IVM), the semi-nude (SN) and Neutral Red group (NR) respectively. In view of future vitrification and IVF, cumulus cells were partially removed (semi-nude) by pipetting to facilitate oocyte handling and future cryoprotectant penetration. Following 30 minutes incubation with 15µg NR/ml maturation medium and a subsequent 1h washout period (NR group), all 3 groups were subjected to routine IVP (cultured under oil for 8 days). Cleavage and blastocyst rate were observed at respectively 2 and 8 days post-insemination. Developmental competence data were analyzed using a binary logistic regression including treatment as fixed factor and replicate as random factor (IBM SPSS version 22). Although there is a significant difference in cleavage (75 vs 55.8%) and blastocyst (36 vs 20.9%) ratio between the LAB CTRL and SN group, our results demonstrate that semi-nude oocytes still have an acceptable fertilization rate that can definitely be improved. However, oocytes from the NR-group significantly failed to cleave (42.9%) and develop to the blastocyst stage (2.4%) as compared to the CTRL and SN group. In conclusion, Neutral Red clearly affects cleavage and blastocyst formation of semi-nude oocytes in the above used conditions and therefore is not suitable for semi-nude oocyte viability assessment.

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Influence of caffeine supplementation prior to *in vitro* maturation on bovine oocyte developmental capacity

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Keywords: caffeine supplementation, cattle, *in vitro* maturation, oocyte.

Although, *in vitro* oocyte maturation (IVM) is common practice in the cattle industry, it is known that the mechanisms involved in meiotic resumption begin in a non-physiological way. The cyclic AMP pathway plays an important role in resumption of meiosis. When cumulus-oocyte-complexes (COC) are mechanically released from the follicle to perform IVM, cAMP levels in immature COC rapidly decrease, which in turn triggers meiosis continuation. It has been proposed that modulation of cyclic AMP prior to IVM can increase bovine blastocyst rates *in vitro*. Caffeine is a non-specific competitive phosphodiesterases (PDE) inhibitor and can inhibit meiotic resumption of oocytes due to maintenance of cAMP levels. It has been reported that gamete treatment with caffeine can increase developmental potential. The present study evaluated the influence of pre-IVM culture in the presence of different concentrations of caffeine on meiotic progress, developmental rates and blastocyst cell numbers. Bovine ovaries were collected from a local abattoir. A total of 4378 cumulus-oocyte-complexes were obtained by slicing. Four different concentrations of caffeine (Merk, Darmstadt, Germany) were used during slicing, searching and 2h pre-IVM culture: 1, 5, 10, 20 mM. A control group, using 2h pre-IVM without caffeine (0mM) and a standard control were also included. After pre-IVM, oocytes were washed and cultured for 24h *in vitro* without caffeine. Following maturation, oocytes were fertilized *in vitro* for 19h and zygotes were cultured *in vitro* for eight days to assess embryo development. Some oocytes were fixed in 2% glutaraldehyde at 9, 20 and 24 h after IVM. Hoechst staining was performed to evaluate nuclear status. Cleavage and blastocyst formation rates were evaluated. Expanded blastocysts from all treatments were submitted to differential staining. One-way ANOVA from R software was implemented to evaluate differences in progression through meiosis, cleavage and blastocysts rates and blastocyst cell numbers. Caffeine maintained the meiotic arrest after 9h IVM in a concentration dependent manner (GV: 100 ± 0.0%, 61.3 ± 21.3%, 40.7 ± 5.4 %, 36.2 ± 11.4% 11.9 ± 6.3%, 28.5 ± 10.0% for 20, 10, 5, 1, 0 mM and standard, $p<0.05$, mean ± SEM). Cleavage (57.7 ± 4.9%, 56.5 ± 3.8%, 62.7 ± 3.2%, 52.5 ± 5.1%, 54.4 ± 6.0%, 60.3 ± 2.3% for 0, 1, 5, 10, 20 mM and standard, $p>0.05$, mean±SEM) and blastocyst rates (26.2 ± 3.0%, 14.9 ± 2.8%, 22.4 ± 3.8%, 23.7 ± 2.1%, 21.4 ± 4.1%, 26.6 ± 2.4% for 0, 1, 5, 10, 20 mM and standard, $p>0.05$, mean ± SEM) and number of cells (ICM: 46.0 ± 4.1, 43,2 ± 3.7, 61.4 ± 7.8, 53.0 ± 6.5, 49.4 ± 5.6, 50.0 ± 4.4; TE:111.6 ± 13.6, 115.4 ± 7.8, 106.4 ± 3.5, 102.6 ± 8.3, 118.4 ± 14.6, 119.6 ± 11.7 for 0, 1, 5, 10, 20 mM and standard, $p>0.05$, mean±SEM) did not differ significantly among *in vitro* treatments. Although caffeine supplementation prior to IVM delayed resumption of meiosis, it did not affect subsequent embryo development and quality.

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Temporal pattern of steroid hormone concentrations during *in vitro* maturation of bovine oocytes

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Keywords: cattle, *in vitro* maturation, steroid hormones.

Present in vitro maturation (IVM) systems do not completely mimic the in vivo situation resulting in oocytes of reduced quality. Steroid hormones are regulators in the fine-tuned mechanism of follicular and oocyte maturation and development. During final maturation a switch from estradiol dominance to progesterone dominance within the follicle is well-described. This change is accompanied by the resumption of meiosis and results in the maturation of the oocyte. It also suggests the important role of these hormones in this process. Aim of the study was to determine the temporal pattern of steroid hormone concentrations in the IVM medium of bovine cumulus-oocyte-complexes (COC) supplemented with different gonadotropin concentrations. COC were obtained from abattoir-derived ovaries and were matured in medium TCM 199 (Tissue Culture Medium 199) supplemented with three different compounds of gonadotropins employing a standard protocol. The three combinations of gonadotropins were: 1. equine (eCG) and human chorionic gonadotropin (hCG), 2./3. follicle-stimulating hormone (FSH) and luteinizing hormone (LH), each in two different concentrations 0.05 IU or 0.01 IU, and 4. without any supplementation of gonadotropins. Groups of 30 COC were matured for 24 hours at 39°C and 5% CO^2 without oil overlay. 17β-estradiol (E2) und progesterone (P4) were measured in maturation medium before use (0h, control) and after specific time points of IVM via radioimmunoassay (RIA). So far, the following results could be obtained. *Treatment 1: TCM with eCG and hCG:* P4 and E2 could not be detected in the control medium (0h). During IVM, P4 concentrations increased in the medium (4h: 3.3 ± 1.0 ng/ml; 8h: 6.2 ± 3.3 ng/ml; 12h: 6.5 ± 2.0 ng/ml; 16h: 6.8 ± 1.1 ng/ml; 20h: 7.3 ± 1.8 ng/ml; 24h: 10.4 ± 1.6 ng/ml), whereas the E2 concentrations stayed similar (4h: 52.8 ± 12.1 pg/ml; 8h: 54.6 ± 7.9 pg/ml; 12h: 63.8 ± 15.2 pg/ml; 16h: 54.2 ± 16.3 pg/ml; 20h: 77.1 ± 40.1 pg/ml; 24h: 74.7 ± 32.4 pg/ml). Treatment 2/3: *TCM with FSH and LH:* Supplementation of 0.05 IU each, E2 concentrations stayed at the same level as with eCG and hCG (E2 0h: 6.2 ± 5.7 pg/ml, washing medium: 26.0 ± 10.8 pg/ml, after 24h: 59.7 ± 20.1 pg/ml). With the supplementation of 0.01 IU each, P4 and E2 concentration also stayed at the same level as with eCG and hCG (E2 0h: 3.3 ± 3.2 pg/ml, washing medium: 19.6 ± 4.2 pg/ml, after 24h: 58.4 ± 24.1 pg/ml, P4 0h: ≤0.25 ng/ml, washing medium: 0.3 ± 0.1 ng/ml, after 24h: 17.3 ± 3.5 ng/ml). *Treatment 4:* After 24 h of IVM *without gonadotropins* the following hormone concentration could be detected: E2: 129.4 ± 88.8 pg/ml and P4: 6.7 ± 0.8 ng/ml. During IVM, the temporal pattern of E2 and P4 did not correspond with the pattern during final maturation in vivo. This underlines that present conditions of IVM do not reflect the in vivo situation and require further optimisation.

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Time-lapse analysis of early cleavage in bovine embryos produced in serum-free medium

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Keywords: cattle, embryo cleavage, IVP, time-lapse cinematography, viability.

Two decades ago, early cleaving embryos were considered as developmentally more competent than slow cleaving embryos. But this theory has been challenged, since moderately developing embryos have decreased chromosomal abnormalities, normal *H19* and *Snrpn* imprint maintenance and potentially higher pregnancy rates. We want to analyze the incidence of chromosomal instability (CIN) in bovine cleavage stage embryos and relate this with developmental kinetics. Hence, we need a culture system allowing individual identification and selection of cleavage stage embryos for single cell analysis (SCA). In this preliminary study, we used time-lapse cinematography (TLC) as a non-invasive tool to describe kinetics and to use timing of early cleavages as a parameter predictive of blastocyst development. Bovine embryos were produced from immature oocytes derived from slaughtered cattle. Oocytes were matured in 500 µL TCM199 supplemented with 20 ng/mL epidermal growth factor (EGF). After in vitro fertilization with frozen-thawed bull semen, 9 presumed zygotes (7 replicates) were cultured in a WOW dish in 30 µL Synthetic Oviduct Fluid (SOF) supplemented with 0.4% BSA, 5 µg/mL insulin, 5 µg/mL transferrin and 5 ng/mL selenium (ITS), covered with mineral oil. In total, 63 zygotes were observed with TLC (Primo Vision®, VitroLife, Göteborg, Sweden), and images were taken every 15 min for up to 90 hours post insemination (hpi). At 192 hpi, blastocyst formation was set as endpoint. Timing of the first (t_1; cleavage into 2-cell stage) and second mitosis (t_2; cleavage into 4-cell stage) and the interval time between those two parameters were analyzed ($t_{\Delta 1\text{-}2}$). The median observation of each parameter was set as a threshold value (t_1 29.00h; t_2 38.83h; $t_{\Delta 12}$ 10.87h). All data were analyzed using a binary logistic regression model. Significantly more embryos reached the blastocyst stage when they cleaved before 29.00h into 2-cell stage or before 38.83h into 4-cell stage (48.3% and 51.2%, respectively), compared to embryos with a later first or second mitosis (16.1% and 18.9%, respectively) ($P<0.05$). Furthermore, when the interval between the first and second mitosis ($t_{\Delta 12}$) was shorter than 10.87h more embryos reached the blastocyst stage (42.3%), compared to a longer interval $t_{\Delta 12}$ (21.1%) ($P<0.01$). This indicates that timing of early cleavage is predictive for further developmental potential, which is confirming earlier studies (Van Soom *et al*., Theriogenology, 38:905-919, 1992; Grisart *et al*., J Reprod Fertil, 101:257-264, 1994). It is however the first time embryos have been cultured in WOW-dishes in serum-free medium and monitored using TLC. WOW dishes offer the advantage of small group culture with individual embryo follow-up, which allows specific embryo selection at any time of the development. Next, we want to identify CIN in embryos with particular cleavage patterns using TLC with SCA and eventually transfer embryos with high and low predicted viability.

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3D visualization of bovine oocyte *in vitro* maturation by confocal laser scanning microscopy

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Keywords: bovine, fluorescence microscopy, oocyte maturation.

Regular nuclear and cytoplasmic oocyte maturation is a prerequisite for normal fertilization and embryo development. Improvement of *in vitro* maturation systems is a central issue in veterinary and human reproductive medicine. Thereby, better microscopic visualization of cellular processes and structures is essential for further extending our rudimentary knowledge and understanding of mammalian oocyte maturation. We used three-dimensional multicolor fluorescence microscopy to investigate critical steps of meiotic maturation *in vitro*. The primary aims of this study were to simultaneously gain information on the meiotic spindle apparatus, on the kinetics of meiotic progression, on the dynamic changes of the cytoskeleton and on the meiotic failures and aberrations. In cattle, the cumulus oophorus is considered to play an essential role for normal oocyte maturation. This makes direct microscopic live cell imaging of the oocyte rather difficult. Thus, cumulus-enclosed grade I and II oocytes were collected from slaughterhouse ovaries and allowed to mature for variable times from 0 to 28 hours *in vitro*. The oocytes were denuded and then fixed with formaldehyde in a microtubule-stabilizing buffer in such a way that the three-dimensional cell architecture was maintained, and were stained for DNA, microtubules and f-actin microfilaments. In addition, serine 10-phosphorylated histone H3 was used as a marker for chromosome condensation and the spindle midbody. For three-dimensional imaging of the oocytes *in toto*, confocal serial sections were captured at 1 µm distance using a 40x objective (NA = 1.3). For imaging details we used a high spatial sampling density (pixel size 50 x 50 nm, z-step size 200 nm) and image restoration by maximum likelihood estimation (MLE) deconvolution. A collection of more than 500 confocal image stacks gives a clearer and more detailed view of completion of meiosis I and progression to metaphase II. Qualitative and quantitative data analyses provide a basis for studies on molecular mechanisms e.g. on the dynamic localization and function of potential key proteins. Important is the detection of anomalies of meiosis I that result in irregular genomic configurations in the zygote: Main findings were (i) the failure of first polar body extrusion as a consequence of incorrect positioning or orientation of the meiotic spindle and (ii) lagging chromosomes, chromatin bridges and incomplete polar body cytokinesis due to irregular spindle formation, chromosome congression and segregation. 3D fluorescence microscopy allows to exactly determine the stage of oocyte meiosis and to diagnose fatal aberrations of meiotic maturation. Thus, high speed imaging systems could be used to test and to improve oocyte isolation methods and in vitro maturation systems.

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Concentration of procaine and exposure time influence *in vitro* fertilization rate in the equine

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Keywords: horse embryo, *in vitro* fertilization, procain exposure.

Most wild equids are currently endangered or threatened, as mentioned in the Red List of the International Union for the Conservation of Nature and several domestic horse breeds are at risk of extinction. Genome resource banking requires cryoconservation of semen, oocytes and/or embryos. Embryo production in equids is limited *in vivo*, since routine induction of multiple ovulation is still ineffective. Embryo production *in vitro* allows the production of several embryos per cycle that could easily be frozen owing to their small size. Intracytoplasmic Sperm Injection (ICSI) has been widely adopted to generate horse embryos *in vitro*, however ICSI is time-consuming and requires expensive equipment and expertise in micromanipulation. We have established an efficient *in vitro* fertilization (IVF) technique in the equine (Ambruosi et al., 2013 Reproduction, 146: 119-133) but IVF zygotes have a low developmental competence. Incubation of gametes with procaine, necessary for induction of sperm hyperactivation, may have a deleterious effect on embryos quality. Our objective was to increase the developmental competence of the IVF zygotes by decreasing procaine concentration or exposure time. Immature cumulus-oocyte complexes were collected from slaughtered mares in a local slaughterhouse, cultured for 26 hours in an *in vitro* maturation medium and pre-incubated for 30 minutes in oviductal fluid collected from slaughtered females. Fresh sperm was collected, diluted to $10x10^6$ spermatozoa/ml, incubated for 5 hours in a capacitating medium and diluted to $1x10^6$ spermatozoa/ml. Spermatozoa were then added procaine (1mM or 5mM) and co-incubated with oocytes for 2, 4 or 18 hours. Zygotes were cultured in DMEM-F12 for 48 hours post-IVF, fixed and analyzed. In experiment 1, spermatozoa were added 5mM procaine and co-incubated with oocytes for 2 hours *vs* 18 hours. The percentage of zygotes 48 hours post IVF was higher for 18 hours co-incubation (62%, 13/21) than for 2 hours (0%, 0/22) (Chi2 test $p<0.05$). In experiment 2, spermatozoa were added 5mM procaine and co-incubated with oocytes for 4 hours *vs* 18 hours. The percentage of zygotes 48 hours post IVF was similar for 18 hours (44%, 7/16) and 4 hours co-incubation (32%, 6/19) (Chi2 test $p>0.05$). In experiment 3, spermatozoa were added 5mM *vs* 1mM procaine and co-incubated with oocytes for 18 hours. The percentage of zygotes 48 hours post IVF was higher for 5mM procaine (48%, 13/27) than for 1mM (19%, 5/26) (Chi2 test $p<0.05$). In the 3 experiments, zygotes contained at least 2 highly decondensed pronuclei, pronuclei decondensation being the first step of embryo development. We also observed 2 cleaved embryonic structures in the group 5mM during 18 hours, but the quality of these embryos was poor. In conclusion, decreasing procaine concentration or exposure time influence IVF rate and doesn't improve equine embryo quality.

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Reproductive success in interbred ewes after fresh embryo transfer

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Keywords: MOET, pregnancy, Romanov sheep, Turkish native sheep.

Estrous response and ovulation rate of Turkish Native Sheep (N; n=27) and the prolific Romanov crossbred breed (NR; n=22) for the use as recipients in embryo transfer programs were evaluated in the anoestrus season (April). Estrus of recipient ewes was synchronized with vaginal sponges containing 30 mg FGA for 12 days and ewes received an i.m. injection of 400 I.U. PMSG at sponge removal. Estrus and ovulation rates, time to onset of estrus and site of the ovulation were determined. A total number of 3 Romanov breed donors ewes were superovulated using FSH-p with 200 mg NIH-FSH-P1 (total of 20 ml) (Folltropin-V; Vetrepharm, Canada) applied in 8 decreasing doses of 1.5, 1.5, 1.5, 1.25, 1.25, 1, 1, 1 ml i.m. at 12 h intervals, starting 60 h before sponge withdrawal. Donors received 1ml Estrumate and 100 I.U. PSMG 36h prior to sponge removal and finally an additional 200 I.U. PMSG was injected at sponge removal. Donors were mated with their own breed of rams. Ewes were observed for estrus (d 0) and were surgically flushed by laparotomy 6 or 7 d later to recover embryos. The number of ovulations and transferable embryos were 18.7 and 14.3, respectively. Embryos with Grade I, II and III with the stage of morula to expanded blastocysts were used in twin fresh transfer. The success rate of the synthetic progestagen treatment to establish an estrus out of season (April) was found to be 59.3% and 52.4% for N and NR, respectively. Time between the removal of the sponges and the onset of estrus was similar between the two breeds of recipient ewes (N: 53.06±0.95h and NR: 52.27±1.07h). For the recipient ewes at ET the ovulation rates were found significantly higher ($P<0.05$) in N ewes (1.0±0.00) than NR ewes (0.72±0.14); the ovulation site was mainly located on the right ovary in NR ewes (87.5%) compared to N ewes (42.9%). We transferred embryos as pairs to save number of recipients as advised by Gimenez-Diaz (2012) who indicated that pregnancy success for number of embryos transferred (single versus twin) was similar. Recipient ewes with fertile estrus (estrus accompanied with ovulation) received similar stages of embryos fallowing the laparoscopic measurements (location, number and quality score) of CL in recipients. Pregnancy and embryo survival rates were similar in N (64.3% and 77.8%) and NR (75.0% and 75.0%) ewes. The sex ratio of twin transferred embryos was higher in N (75% male) than those observed in NR (22.7% male) ewes. These preliminary results show that, Romanov crossbreed recipients with a lower ovulation rate and ovulation occurred mostly in right ovary had more overall MOET success (66% vs 57%; no of lambs born/no of embryo transferred) and were found more favourable with more female lambs from fresh embryo transfer compared to Turkish Native Sheep.

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Estradiol route and non-surgical embryo recovery in synchronized Santa Inês ewes

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Keywords: embryo recovery, estradiol benzoate, Santa Inês ewes.

Animal surgery procedures are being progressively restricted worldwide in the context of embryo transfer. In small ruminants, the needs for development of alternative and efficient non-surgical techniques for embryo transfer have been emphasized. Non-surgical embryo recovery is well consolidated in Brazil in cattle and goats, while in sheep it remains a challenge. The aim of this study was to check the efficiency of different ways of estradiol benzoate administration on cervix dilation and embryo recovery in synchronized Santa Inês ewes. A total of 23 pluriparous ewes were subjected to two doses of 37.5 μg d-cloprostenol by intravulvo-submucosal way seven days apart. After the second cloprostenol administration, ewes were checked for estrus at 12 h interval and mated with fertile rams during estrus. After mating, ewes were allocated according to estrous response into two treatment groups for embryo recovery seven days after estrous onset. In T1 (n=11), ewes received 37.5 μg d-cloprostenol and 1 mg estradiol benzoate 16 h before embryo recovery, plus 50 IU oxytocin i.v. 20 min before embryo recovery. In T2 (n=10), ewes received the same protocol as T1, but the way of estradiol administration was intravaginal. All ewes received 2 ml of lidocaine 2% without vasoconstrictor for epidural and 2 ml of lidocaine for contact cervical anesthesia plus acepromazine 1% (1 ml/kg live weight) before cervical passage as previously described in goats (Fonseca et. al.; Small Rumin. Res., 111:96-99, 2013). Qualitative and quantitative data were analyzed by chi-square test and ANOVA respectively with 5% significance. Estrous response after the second cloprostenol administration was 91.3% (21/23). There were no differences (P>0.05) in any parameter evaluated for T1 and T2: successful uterine flushing (90.9% and 80.0%), duration time of embryo recovery (20.3 ± 8.0 and 26.2 ± 5.3min), flushing recovery rate (PBS injected/PBS recovered; 90.1 and 90.5%), average structures recovered (1.0 ± 0.4, 20% viable and 1.4 ± 0.6, 33% viable). Considering that Santa Inês sheep have up to 1.3 lambs we can conclude that it is possible to perform efficient non-surgical embryo recovery in non-superovulated synchronized Santa Inês ewes, regardless the way of estradiol administration.

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Effect of Thymosin on *in vitro* fertilization and developmental competence and quality of pig embryos

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Keywords: IVF, pig embryo quality, thymosin.

Thymosin (TH) is biological active polypeptide released by thym. It plays a vital role in the repair and regeneration of injured cells and tissue. It protects cells from damage and blocks apoptosis (Goldstein et al. 2012, Expert Opin Biol Ther, 12(1), 37-51). In our recent study we demonstrated that supplementation of maturation medium for pig oocytes with synthetic TH increased the number of matured oocytes with lower morphological quality. The aim of the present study was to investigate the effect of supplementation of maturation medium for oocytes with TH on *in vitro* fertilization and developmental competence and quality of pig embryos. Cumulus-oocyte complexes (COCs) were obtained by aspiration from antral follicles of ovaries collected from slaughtered gilts. COCs were selected based on their cytoplasm morphology and cumulus cells appearance and cultured in modified TCM-199 medium supplemented with 0.5 mg/ml of synthetic TH (LipoPharm.pl) (experimental group) or without TH (control group) for 42 h at 39^{o}C and in a humidified atmosphere containing 5% CO_2 in air. After maturation, oocytes were assessed and *in vitro* fertilized (IVF). Semen for IVF was incubated in modified capacitation medium-M-199 for 1 h. Sperm fraction was introduced to the droplets containing oocytes and next gametes were coincubated for 4 h in modified TCM-199 medium. Presumptive zygotes were cultured *in vitro* for 144 h in NCSU-23 medium under the conditions stated above. Embryo quality criteria were cleavage, morula and blastocyst rates, total cell number per blastocyst and degree of apoptosis assessed by TUNEL. The results were analyzed statistically with Chi-square test. Treatment of oocytes with TH during culture slightly increased the ratio of matured oocytes (95/103, 92.3%) compared to the control group (134/150, 89.3%; no significant differences) cultured without TH. After IVF cleavage, and development to the morula and blastocyst stage, based on number of cleaved embryos, were not different between experimental (29.5, 71.4 and 32.1%, respectively) and control (25.4, 50.0 and 29.4%, respectively) group. The mean number of cells per blastocyst in experimental and control group was comparable (40.4 and 39.9; respectively). The mean number of apoptotic nuclei and apoptotic index was 0.67 and 1.66 in the experimental group and was significantly lower ($P<0.05$) than in the control group i.e. 1.66 and 4.35. In conclusion, the culture oocytes in a medium with TH supplementation had a positive effect on quality pig IVF blastocyst since they had a significant lower incidence of apoptosis. However, further studies are required to determine the competence of porcine blastocyst recovered from oocytes matured with TH for *in vivo* development.

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A200E OPU-IVP and ET

A retrospective study of *in vitro* embryo production from high genetic merit cows using unsorted or X-sorted sperm in a commercial program

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Keywords: breeding program, cattle, IVP, semen sexing.

X-sorted sperm can be used in embryo transfer programs to produce female progeny (Kaimio et al., Theriogenology 80, 950-954, 2013). However, X-sorted sperm is generally used in heifers as cow insemination results in lower numbers of transferable embryos (Hayakawa et al., Theriogenology 71, 68-73, 2009). We hypothesized that breeding programs based on IVP with X-sorted sperm may be a promising alternative. The aim of this study was to compare *in vitro* embryo production in cows using X-sorted or unsorted semen under commercial conditions performed at the Biotechnology MIDATEST Station located in Denguin, South West, France. Three to fifteen years old Holstein cows (n=26) and 16-22 months old heifers (controls: n=17) were used in an OPU-IVP program. Donor cows were stimulated with decreasing pFSH doses (Stimufol; Reprobiol, Liège, Belgium) twice daily during 3 days, (total dose: 350 µg for cows and 250 µg for heifers). Cumulus oocyte complexes (COCs) were collected by OPU 12 to 24 h after the last FSH injection and *in vitro* matured using a standard IVM protocol. Oocytes were fertilized with frozen–thawed unsorted (cows and heifers) or X-sorted (cows) sperm in modified Tyrode's bicarbonate buffered solution medium (fert-TALP) using different non pre-tested bulls (n=55). Presumptive zygotes were cultured in SOF medium (Minitub, Tiefenbach, Germany) plus 1 % cow serum up to Day 7 at 38.5 °C in 5% CO2, 5% O2 and 90% N2 atmosphere with maximum humidity. OPU/IVP was repeated one to 13 times (2.5 ± 2.6) for each donor cow or heifer. Grade 1 blastocysts and expanded blastocysts according to IETS classification were recorded on days 6.5 and 7. Viable embryos were frozen or transferred fresh. Embryo production was analyzed with ANOVA and blastocyst yield by Chi-Square. From 18 OPU sessions in heifers, a total of 168 COCs (9.3 ± 4.7 per session) were processed for *in vitro* maturation, and 5.4 ± 3.9 Grade 1 (G1) embryos were produced per session. In cows 42 sessions were performed with unsorted semen and 44 with X-sorted semen, 13.1 ± 9.6 and 8.9 ± 4.9 oocytes ($p<0.05$) were collected; 7.7 ± 5.5 and 4.1 ± 2.9 G1 embryos were produced, respectively ($p<0.05$). The mean embryo development rate (total number of G1 embryos / number of oocytes entering maturation process) was 59.1% (unsorted semen) and 46.3% (X-sorted semen; $p<0.05$). Although the number of collected oocytes was different, there were no differences in presumptive female embryos produced per session assuming a sex ratio of 90% (3.7 embryos per session) with sorted semen and a sex ratio of 50% (3.9 embryos per session) when using unsorted semen. In conclusion, our work confirmed the efficacy of OPU-IVP techniques to produce grade 1 embryos using X-sorted in high genetic merit cows. Furthermore this technique allows to get female calves based upon a lower number of recipients.

A201E OPU-IVP and ET

Presence of L-carnitine during maturation improves efficiency of fertilization in porcine oocytes

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Keywords: *in vitro* fertilization, *in vitro* maturation, L-carnitine, pig embryo.

The lipid-rich pig oocytes might be an excellent model to understand the role of fatty acid metabolism during oocyte maturation, their subsequent monospermic fertilization and preimplantation embryo development. Recently, it was described that L-carnitine stimulates mitochondrial oxidation of fatty acids and increases energy supply of mammalian oocytes. The aim of the study was to characterize the effect of L-carnitine during maturation on the efficiency of fertilization of porcine oocytes with different meiotic competence. Cyclic sows, checked for the ovarian cycle status, were used as oocyte donors. Meiotically more competent (MMC) and meiotically less competent (MLC) oocytes were isolated either from medium (6–9 mm) or small follicles (<5 mm). They were matured separately in IVM medium supplemented with 0, 4 and 10 mM L-carnitine (Sigma-Aldrich Co., Prague, Czech Republic) and fertilized by frozen-thawed spermatozoa of a boar proven in the IVF system using standard protocols (Hulinska et al. 2011, Anim Reprod Sci, 124: 112–117). The presumptive zygotes were incubated in PZM-3 medium (Yoshioka et al. 2002, Biol Reprod, 66: 112–211) for 15 h, fixed in 2.5% aqueous glutaraldehyde solution (v/v), stained with bisbenzimide-33258 Hoechst (Sigma-Aldrich Co., Prague, Czech Republic) and examined by epifluorescence at a magnification of 400 ×. The proportion of penetrated oocytes from the inseminated ones and proportions of monospermic and polyspermic oocytes from the penetrated ones were assessed. Total efficiency of fertilization (%) of oocytes was calculated according to the formula (ratio of monospermic oocytes (n) to inseminated oocytes (n) × 100). The results were statistically analysed by the ANOVA procedure using the Chi-square test. In MMC-oocytes total efficiency of fertilization increased (51.1, 54.3 and 57.6%) when the oocytes were matured with 0, 4 and 10 mM L-carnitine. Similarly in MLC-oocytes, total efficiency of fertilization increased (42.1 vs 48.8%) in oocytes matured with 4 mM L-carnitine compared to those matured without L-carnitine. On the other hand, total efficiency of fertilization decreased when MLC-oocytes were matured with 10 mM L-carnitine (37.9%). It can be concluded that supplementation of medium with L-carnitine during maturation positively influenced fertilization efficiency of porcine oocytes independently of their meiotic competence. However meiotically more competent oocytes were more capable of utilizing L-carnitine in comparison with meiotically less competent porcine oocytes in which the abundance of L-carnitine had a negative effect on fertilization.

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A202E OPU-IVP and ET

Effects of resveratrol supplementation during *in vitro* maturation and *in vitro* fertilization on developmental competence of bovine oocytes

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Keywords: bovine oocytes, *in vitro* fertilization, *in vitro* maturation, resveratrol.

Resveratrol (3,4',5-trihydroxystilbene) is a phytoalexin - isolated from various plant species, particularly grapevine peel. Recently, resveratrol gained scientific interest because of its strong antioxidant effects it may have health benefits, including protection against cardiovascular diseases. In addition, it has been shown to increase lifespan in several species and activates the SIRT1 gene. The aim of this study was to investigate its effects in bovine early embryo development. We employed three different resveratrol concentrations during *in vitro* maturation (IVM) and *in vitro* fertilization (IVF). Bovine oocytes (n=1648) were collected from slaughterhouse ovaries and subjected to IVM medium supplemented with 0.2µM, 1µM, and 20µM Resveratrol® (Sigma-Aldrich, Buchs, Switzerland) for 24 h followed by IVF with the same concentrations of resveratrol for 19 h. IVM and IVF medium without resveratrol (control) and DMSO supplementation as vehicle control were included in this experiment. Presumptive zygotes were cultured *in vitro* until day 8 to assess embryo development. Maturation rates, cleavage and blastocyst formation were determined. Maturation rates did not differ significantly (0.2µM: 64.2 ± 7%; 1µM: 82.3 ± 4%; 20µM: 68.8 ± 2%; control: 74.6 ± 5% and vehicle control: 70.2 ± 6%, respectively, $p \leq 0.05$) did not differ dramatically. Oocytes cultured in 1µM resveratrol supplemented maturation medium showed distinct detachment of cumulus cells. Cleavage was reduced in the 0.2µM and 20µM group (0.2µM: 44.21 ± 2%; 1µM: 58.4 ± 3%; 20µM: 40.9 ± 5%; control: 56.6 ± 2% and vehicle control: 55.2 ± 6%, respectively, $p \leq 0.05$). Blastocyst development was impaired in the low and high resveratrol concentration group compared to the other groups (0.2µM:11.3±1%;1µM: 28.4 ± 6%; 20µM: 8.2±4%; control: 22.7 ± 4% and vehicle control: 20.8 ± 2%, respectively, $p \leq 0.05$). These preliminary results indicate that very low and high concentrations of resveratrol impair the development to the blastocyst stage. In conclusion, a 1µM resveratrol supplementation during IVM and IVF seems to improve the developmental competence of oocytes, which is reflected not only in elevated blastocyst rates but also in the higher degree of expansion of cumulus cells after IVM and the maturation rates.

A203E OPU-IVP and ET

Interpretation of equine *in vitro* produced embryo morphology

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Keywords: embryo, Hoechst, horse, ICSI.

To better understand the correlation between equine *in vitro* produced embryo morphology and nuclear status. Oocytes were recovered from abattoir-derived ovaries, matured *in vitro,* subjected to conventional ICSI and cultured *in vitro*. Assessment of nuclear status by staining with Hoechst 33258 and using fluorescent microscopy was performed at the following times after ICSI: Group A) 20 hours to evaluate pronuclear (PN) status; Group B) Day 2, 3, 4, or 7 to determine nucleus number and correlation of morphological cleavage; and Group C) Day 7 to 11 to determine blastocyst development. Only normal nuclei were included in the number of nuclei recorded; nuclei with signs of degeneration (vacuolization, condensation or fragmentation) were disregarded. Confirmed blastocysts contained > 64 normal nuclei and showed arrangement of an outer rim of nuclei in a presumptive trophoblast layer. Two Day-9 presumptive blastocysts were transferred to the uterus of a recipient mare to evaluate viability. A total of 109 oocytes were subjected to ICSI in groups A and B. In Group A, the rate of PN formation was 43%. In Group B, 64% demonstrated apparent morphological cleavage but only 17% had ≥ 2 normal nuclei on staining and only 6.5% had a number of nuclei that matched the number of visible blastomeres and were appropriate for age. The other stained embryos that appeared cleaved morphologically possessed only degenerated nuclei or were completely anuclear. In Group C (n = 138 injected oocytes), 17 embryos were presumed to have developed to the blastocyst stage based on morphological criteria. Of these, 7 were confirmed blastocysts by staining and 8 were degenerating embryos. One embryo, presumed to be degenerated, was also revealed to be a blastocyst. Notably, as uncleaved oocytes were placed in a separate droplet at Day 4 but were kept in culture, we could evaluate changes in these oocytes over time. Several known uncleaved oocytes increased in diameter on Day 9, which on simple morphological evaluation, could have led to mistaken classification as blastocysts. Transcervical transfer of two Day-9 presumptive blastocysts to the uterus of a recipient resulted in 2 embryonic vesicles detected on Day 14 after ICSI. The smaller vesicle was manually reduced and the remaining vesicle developed normally and is > 250 days gestation. Overall in Group C, including the two transferred embryos, the rate of confirmed blastocyst development per injected oocyte was 7.2%. To the best of our knowledge, this is the first report documenting the morphology and DNA staining of equine *in vitro*-produced blastocysts vs. blastocyst-like structures. Our findings reinforced the importance of removing uncleaved oocytes to limit uncertainty in later assessments of blastocyst development, and of staining embryos for DNA to definitively establish blastocyst development.

A204E OPU-IVP and ET

Transfer of cattle embryos produced with sex-sorted semen results in impaired pregnancy rate

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Keywords: cattle, embryo transfer, pregnancy, sex-sorted semen.

This study compared the pregnancy rates after transfer of day-7 *in vivo* embryos produced either with conventional or sex-sorted semen from numerous bulls commercially available and extensively used. In addition, mortality of calves born from sexed embryos and conventionally produced embryos was studied. The data consisted of 12,438 embryo transfers, of which 10,697 embryos were produced using conventional semen (CONV embryos) and 1,741 using sex-sorted semen from 97 bulls (SEX embryos), predominantly of Ayrshire and Holstein breeds. Quality codes of embryos were similar in both groups. Of the CONV embryos, 27.4% were transferred fresh, the proportion being 55.7% for SEX embryos. Recipient properties (breed, parity, number of previous breeding attempts and interval from calving to transfer) were similar for both embryo types, heifers representing 57.8% of recipients in the CONV group and 54.8% in the SEX group. Recipients that were not inseminated or did not have a new embryo transferred after the initial one, and had a registered calving in fewer than 290 days after the transfer, were considered pregnant. Data were analyzed with IBM SPSS Statistics, Version 21.The effects of sexing protocol, embryo type (fresh vs. frozen), developmental stage, quality and breed of embryo as well as parity (heifer vs. cow) and breed of a recipient on conception were analyzed using binary logistic regression. Pregnancy rate for recipients receiving CONV embryos was 44.1% and for those receiving SEX embryos 38.8%. The odds ratio for pregnancy in recipients receiving CONV embryos was 1.34 compared with SEX embryos ($P<0.001$). Other factors affecting the pregnancy rate were embryo quality ($P<0.001$), being highest for grade 1 (CONV 45.2%, SEX 42.8%) and lowest for grade 3 (CONV 29.2%, SEX 22.2%) embryos, and developmental stage of an embryo ($P=0.038$). Transfer of earlier developmental stages, i.e. compact morulas, resulted in lower pregnancy rates than transfer of later stages. Also recipient parity affected pregnancy rate ($P<0.001$), the odds ratio for pregnancy for heifers was 1.18 compared with that for cows. There was no effect of the breed on pregnancy rate, neither of an embryo nor of the recipient. The proportion of female calves was 49.6% and 92.3% in CONV and SEX groups, respectively. Calf mortality was 9.0% and 8.9% in CONV and SEX groups, respectively. Mortality of female calves was similar in CONV and SEX groups, 6.6% and 7.7%, respectively. For male calves, mortality was 9.2% in the CONV group but significantly higher, 16.0% ($P<0.05$), in the SEX group. This study showed that transfer of embryos produced with sex-sorted semen decreased the pregnancy rate by about 12% compared with embryos produced using conventional semen. Mortality of male calves born from SEX embryos was higher than for those born from CONV embryos.

A205E OPU-IVP and ET

Supplementation of culture medium with foetal calf serum or insulin – transferrin – selenium affects the integrity of equine oviduct explants

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Keywords: foetal calf serum, horse, insulin, IVP, oviduct explants, selenium, transferring.

Equine oviduct explants provide an excellent tool to unravel embryo-maternal interactions. They can be cultured *in vitro* for several days in DMEM F12 and serum whilst remaining functionally intact and highly differentiated. However, dark cell degeneration (DCD) has been observed inside the explants (Nelis et al. 2014 RFD 26 954-966). Since serum has been reported to negatively affect cell and embryo culture (Fernandez-Gonzalez 2004 PNAS 101 5880-5885), we aimed to assess the effect of serum and the serum replacer insulin-transferrin-selenium on the prevalence of DCD, ciliary activity, membrane integrity and ultrastructure of equine oviduct explants. Oviducts ipsilateral to the ovulation side were gathered from mares in the early postovulatory stage. Oviduct explants were harvested by scraping and cultured for 6d in 50 µl drops under oil in 5% CO_2 in air in DMEM/F12 (control; Invitrogen, Merelbeke, Belgium), in DMEM/F12 with 10% foetal calf serum (FCS; Greiner Bio-one, Wemmel, Belgium) or in DMEM/F12 supplemented with 5 µg/ml insulin, 5 µg/ml transferring and 5 ng/ml selenium selenite (ITS; Sigma, Schnelldorf, Germany). Three replicates of 60 droplets per condition were performed. With an inverted microscope, every 24h, the percentage of explants with dark zones and the percentage of explants showing ciliary activity were determined. In addition, the percentage of membrane-damaged cells was determined using Trypan blue (Sigma-Aldrich, Diegem, Belgium). At d0, 3 and 6, ultrastructure was assessed by TEM. To compare DCD prevalence, ciliary activity and membrane integrity, binary logistic regression was implemented (SPSS 21 for windows; SPSS IBM, Brussels, Belgium). During the first two days, the prevalence of DCD was significantly lower in the FCS group (36%), when compared to ITS (68%, $P<0.0005$) and the control (67%, $P<0.0005$), indicating an initial protective effect of FCS. From d3 on, significantly more DCD was observed in the presence of ITS and FCS (87% resp. 92%, $P<0.0005$) compared to the control (81%). FCS and to a lesser extent ITS seem to sustain the percentage of explants showing ciliary activity (97%, $P<0.0005$ and 94%, $P<0.0005$) compared with the control (87%). In all groups, as shown by Trypan blue, the explants consisted of >98% membrane intact cells ($P=0.9$). No qualitative differences in the development of DCD was detected by TEM. The outer surface of explants in all groups was highly differentiated and intact. In conclusion, without affecting morphology, components of FCS, which may be depleted after 2 days of culture, turn out to partly protect while ITS enhances the development of DCD. Furthermore, FCS and ITS seem to preserve ciliary activity. Since the toxic margin of insulin and transferrin, but not of selenium, is far above the applied levels in our culture system, amongst others, selenium may play a role in the development of DCD. Further research is needed to unravel the exact cause in the development in DCD in oviduct explants.

A206E OPU-IVP and ET

Reproductive response of prolific breed and its crosses in intrauterine insemination program

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Keywords: conception, laparoscopic AI, Romanov sheep, synchronization, Turkish native sheep.

The success of laparoscopic AI (LAI) depends on events and factors that interrelate in a complex way. Once the selection and preparation of the ewe have been accomplished, one of the most important steps in the program is the successful synchronization of the ewe to deliver good quality ova to the site of fertilization at a specific time. However, a considerable variation in success rate exists when using this technology whereby conception rates range from 10 to 85%. A major and highly consistent finding from the studies reported showed to be a major difference among the different ewe breeds with respect to pregnancy rate after LAI regardless of the source of that semen. These results confirm the importance of the breed and therefore possible reasons for this effect needs to be elucidated. In this study, we aimed to investigate the reproductive performance of yearling prolific Romanov breed and its half and quarter crosses with Turkish native breeds in a LAI program conducted during the breeding season. In addition, to breed effect we also examined vaginal electrical resistance (VER) values which was reported by the previous researches (Bartlewski et al., 1999; Rezac, 2008) that ewes with lower VER, which means higher estrogen levels. A total number of 30 ewes, equally distributed for each genotype (Romanov: 10, F1 Romanov crosses (F1): 10 and quarter Romanov (Q breed): 10) were included in the experiments. All animals were treated with a vaginal sponge containing 30 mg fluorgestone acetate (FGA; Chrono-gest, Intervet, MSD, Turkey, for 12 d. Immediately following sponge removal, ewes received an injection of 500 IU, i.m. eCG. An experienced laparoscopic AI operator performed the inseminations using fresh diluted semen (100 x 10^6 motile spermatozoa/0,4ml) at 52-55h after sponge removal. The animals were screened for estrus beginning at 24 h after sponge removal and continuing up to 57 h. Animals that did not show any mating marks by 57 h were not inseminated. Electric resistances of vaginal secretions (VER) were measured with a vaginal probe (DRAMINSKI, Poland) that was gently inserted into the vagina prior to LAI. *Conception rate* was determined by *ultrasound* 40 days after AI. The Romanov breed showed the highest estrus response (83%; $P<0.05$)) and, the F1 (40%) and Quarter Romanov crosses (50%) were found similar estrus rates. Conception rates (CR) were 80%, 75% and 57% for Romanov, F1 and Quarter Romanov crosses, respectively ($P>0.05$). Correlation coefficient between vaginal mucous impedance and conception rates was computed as 0.025 and showed to be not significantly correlated with CR. However, compared to F1 and Q ewes Romanov ewes showed more tight VER values which is possibly related to the variation in the moment of estrus.

A207E OPU-IVP and ET

The application of bovine *in vitro* embryo production technology to develop an in vitro test battery for the screening of estrogenic compounds

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Keywords: 17β-estradiol, bovine IVP, diethylstilbestrol, *in vitro* assays.

The objective of this study was to develop a battery of tests able to identify the two main mechanisms of action of estrogenic compounds: the receptor-mediated mechanism, naturally occurring in hormone-responsive tissues carrying specific receptors (Erα and Erβ) and the direct mechanism through which estrogen and estrogenic compounds bind spindle components and cause a depolymerizing effect on microtubules therefore inhibiting the correct formation of the meiotic spindle. For this purpose two well-known compounds, diethylstilbestrol (DES) and 17β-estradiol (EST), were tested on four different *in vitro* assays: bovine oocyte *in vitro* maturation (bIVM) assay, bovine embryo *in vitro* culture (bIVC) assay and MCF-7 (human breast adenocarcinoma) and BALB/3T3 cell lines (mouse fibroblasts) proliferation and cytotoxicity assays, respectively. For the bIVM assay immature oocytes were aspirated from abattoir ovaries, washed and transferred to oocyte maturation medium, which was supplemented with the test compounds. At the end of maturation the oocytes were denuded, fixed with acetic acid/ethanol (1:3) for 18-24h and stained with lacmoid solution. The completion of meiosis up to the metaphase II stage was considered as the toxicological endpoint. For the bIVC assay, bovine embryos were obtained by IVM and IVF, followed by *in vitro* culture. At day 7 after IVF, embryos were selected at the early blastocyst stage and exposed to test substances from this stage onwards. The toxicological endpoint was the development of embryos up to the expanded hatched blastocyst stage at day 11. For the other two assays MCF-7 cells were cultured in MEM without glutamine and phenol red supplemented with 10% Foetal Bovine Serum (FBS) charcoal stripped, 4 mM α-glutamine and 1 mM pyruvate and BALB/3T3 cells were cultured in DMEM:TCM199 (1:1) supplemented with 10% FBS charcoal stripped. Both cell lines were exposed to test compounds at increasing concentrations. The AlamarBlue® test was performed and data were analysed with a TECAN plate reader (Infinite F200 Pro). Results indicate that only the MCF-7 proliferation assay can detect the receptor-mediated mechanism in the picomolar range of test compounds whereas a cytotoxic effect appeared in both cell lines in the micromolar range of test compounds. Moreover, the bIVM assay can detect the direct mechanism inducing spindle depolymerisation and abnormal nuclear configuration in the range of 1-20 microM. Finally, the bIVC assay does not seem to be informative because only a cytotoxic effect is evident at the highest concentration tested, as for the BALB/3T3 assay. In conclusion, this battery of four tests can allow to discriminate between the two major mechanisms of action of estrogenic and estrogen-like compounds, the receptor-mediated pathway and the direct one.

A208E OPU-IVP and ET

Using progesterone assay before superovulatory treatment in bovine farms

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Keywords: bovine embryos, progesterone, superovulation.

A French ET team had systematically realized progesterone assay for 15 years to help in the decision to start or not the superovulatory treatment for an embryo flushing in farm.Since 2010, 2210 progesterone assays have been done from 1561 females (1 425 heifers; 1119 Holstein, 30 other dairy breeds, 266 dual-purpose breeds and 146 beef breeds) in 665 farms. The blood samples for progesterone assay were performed by the farmer from 4 to 13 days after a reference heat and sent to a hormonology lab (LNCR, Maisons-Alfort). The superovulation protocol (8 FSH injections) was: D-16 to D-8 = reference heat; D-10 to D-3 = blood sample for progesterone assay; D-2 = input of an implant of norgestomet (Crestar®); D0, 8:00 = first FSH (Stimufol®) injection; D4 = 2 AI depending on heat observation; D11 = embryo flushing. The ET team received the quantitative result from the lab 2 to 6 days after the blood sample. A qualitative result was determined: negative for quantitative result inferior to 1.2 ng/mL, positive for results superior to 1.8 ng/mL and dubious between 1.2 to 1.8 ng/mL. The interval between the reference heat and the first FSH (from 8 to 16 days) didn't influence the number of collected embryos. No clear effect of parity (0, 1, 2, 3, 4 or 5 and more) or kind of breed could be shown, due to the great predominance of Holstein heifers (1 125). Among the 2210 progesterone assays, 1961 (89 %) gave a positive result, 114 (5%) a dubious result and 135 (6%) a negative one. Among the planned embryo collections, 108 (5%) were not performed, 42 due to a negative progesterone result, 66 for other different reasons. The mean progesterone level increased significantly from 4 to 6 days after heat, but this increase was no more significant after 7 days. Actually, 70 % of the negative results were all the same followed by an embryo flushing (result received too late, recipient already prepared…). Of course negative progesterone levels, led to significantly ($p<0.0001$) less total and viable embryos collected than for positive ones: respectively 10.3 ± 8.6 and 5.7 ± 5.3 for positive versus 6.1 ± 5.2 and 3.0 ± 3.6 for negative. But, for the positive results, no effect of the level of progesteronemia on the number of collected embryos has been observed. Because of the very low ratio of embryo flushing finally cancelled due to negative result of the progesteronemia (2%), it has been decided to stop the use of systematic progesterone assay.

A209E OPU-IVP and ET

Effect of low oxygen tension on mitochondrial activity in cultured pig embryos

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Keywords: IVF, mitochondrial activity, oxygen tension, pig embryo quality.

Mitochondrial membrane potential (ΔΨm) is a key factor of the normal pre-implantation embryonic development due to high correlation with the cellular energy production. Consequently, alteration of ΔΨm may improve performance of pig embryo produced *in vitro*. Therefore the goal of this study was to check whether decreasing of oxygen tension may influence ΔΨm during *in vitro* development of pig embryos. Pig zygotes were collected surgically from superovulated gilts after flushing the oviducts. Zygotes were cultured in NCSU-23 (North Carolina State University-23) medium at 39°C in an atmosphere containing 5% CO_2 and: (A) 21% O_2, (B) 5% O_2, (C) 2% O_2. Embryos at 2- to 4-cell, 8- to 16 cell and morula stages were selected on days 2, 3 and 4 of culture, respectively. To estimate ΔΨm embryos were labeled with 0.5 μM MitoTracker Orange CMTMRos (Molecular Probes Inc.) for 30 min. at 39°C and subsequently analyzed in LSM 510 META confocal microscope (Carl Zeiss GmbH). The amount of fluorescence emitted from the mitochondria in arbitrary unit which proportional to the ΔΨm were measured. Data were analyzed using one-way analysis of variance and post-hoc Tukey test. For zygotes ΔΨm (mean±standard error of the mean) equals 8.07±1.28 (N=19). In group (A) ΔΨm was: 7.74±1.65 (N=17), 14.28±2.45 (N=16) and 15.1±2.44 (N=17) for 2- to 4 cell, 8- to 16 cell and morula stage respectively. In group (B) ΔΨm was: 9.73±0.96 (N=11, 2- to 4 cell), 24.52±2.37 (N=20, 8- to 16 cell) and 28.3±1.33 (N=18 morula). For group (C), ΔΨm was: 10.15±1.19 (N=21, 2- to 4 cell), 26,45±1.88 (N=13, 8- to 16 cell) and 32.57±1,04 (N=21, morula). In all analyzed groups, at the 2- to 4 cell stage ΔΨm was very low with no differences between groups, while significantly increased later, at 8- to 16 cell and morula stage ($p<0.01$). In conclusion, significant differences between embryos at the same developmental stages cultured in different oxygen tension were detected. Mitochondrial membrane potential for 8- to 16 cell and morula cultured at ambient oxygen tension was lower than that of stage matched embryos cultured in hypoxia conditions. Further investigations regarding the oxygen-sensitive hypoxia-inducible factors expression during *in vitro* cultured of pig embryos under different oxygen tensions are required.

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A210E OPU-IVP and ET

Serum-free *in vitro* culture of equine embryos

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Keywords: embryo development, horse, IVP, serum free in vitro culture.

While bovine embryos are routinely cultured in serum-free conditions since serum culture is associated with the occurrence of the large offspring syndrome, equine embryo culture is still conducted in the presence of fetal calf serum (FCS). In the horse, a negative effect of in vitro culture on the foals has not been observed, but early embryonic loss and development of trophoblast-only pregnancies have been associated with in vitro production of equine embryos (Hinrichs et al, Theriogenology 68:521-529, 2007). Therefore, the aim of this study was to evaluate equine blastocyst development and quality in serum-free culture medium. Equine embryos were produced as reported previously (Smits et al. Reproduction 143:173-181, 2012). Briefly, oocytes were aspirated from abattoir ovaries, matured in DMEM/F12 based medium in 5% CO_2 in air and fertilized by piezo-assisted ICSI. Presumptive zygotes were further cultured in DMEM/F12 supplemented with either 1) 10% FCS, 2) 10% serum replacement (SR, Life technologies, Gent, Belgium) and 5 ng/ml selenium, or 3) 0.4% BSA (Sigma-Aldrich, Diegem, Belgium), 5µg/ml insulin, 5µg/ml transferrin and 5 ng/ml selenium (BSA-ITS) at 38.2°C in 5% O_2, 5% CO_2 and 90% N_2. At day 2.5 cleavage was assessed and at day 9 blastocyst rate was evaluated. Subsequently blastocysts were fixed in 2% paraformaldehyde during 20 minutes and stored in PBS with 0.5% BSA at 4°C until staining. Differential apoptotic staining was performed as described previously (Wydooghe et al, Anal Biochem 416:228-230, 2011) to determine total cell number (TCN), inner cell massa/trophectoderm (ICM/TE) ratio and apoptotic cell ratio (ACR). Cleavage and blastocyst rates were compared using binary logistic regression. Data concerning blastocyst quality (i.e. TCN, ACR and ICM/TE ratio) were analyzed using a mixed-model analysis of variance (SPSS statistics 22). Cleavage rates were similar in FCS (22/29, 75.8%), SR (20/28, 71.4%) and BSA-ITS (22/28, 78.6%). No blastocysts developed in the BSA-ITS. Blastocyst rates were not significantly different between FCS (7/29, 24%) and SR (4/28, 14%) and TCN and ICM/TE were not affected either. However, ACR was significantly higher in SR (4.16 % ± 0.49), when compared to FCS (0.88% ± 0.20 , $p<0.001$). In conclusion, serum-free IVC of equine embryos in the presence of SR does not impair embryonic development, but ACR in the resulting blastocysts is significantly increased, when compared with ACR in blastocysts cultured in the presence of FCS.

A211E OPU-IVP and ET

The effect of dimethylsulphoxide on bovine embryonic development in vitro

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Keywords: cattle, dimethylsulphoxide, embryo development, IVP.

DMSO is routinely used as cryoprotectant or solvent for in vitro production (IVP) of embryos. Based on its high glassforming characteristics it is essential for vitrification but DMSO is also known for its toxicity at higher concentrations. Earlier studies deemed concentrations of up to 0.4% in in vitro maturation and 0.1% in in vitro culture (IVC) as safe with regards to morphological criteria. In the present study, bovine IVP embryos employing standard protocols were exposed to the following DMSO concentrations during IVC: 0% (control group), 0.05%, 0.1%, 0.15%, 0.2% and 0.25%. At day 8 cleavage and developmental rates were recorded. The morphological quality of expanded day 8 blastocyst was assessed with differential cell stainings; live-dead-staining (live-dead ratio) and TUNEL staining (apoptotic index). Fat accumulation was determined by red-oil staining. So far, the following results could be obtained: Cleavage and developmental rates did not differ ($p \geq 0.05$) between embryos of the various groups. Mean cleavage and development rates averaged at 58.3% ± 10.6% and 28.4% ± 9.2% (0 %), 59.5% ± 11.5% and 26.1% ± 7.4% (0,05%), 57.6% ± 6.6% and 21.7% ± 7.1% (0,1%), 58.1% ± 7.8% and 27.8% ± 5.6% (0,15%), 56.6% ± 7.3% and 24.5% ± 7.0% (0,2%), 56.3% ± 10.9% and 23.5% ± 9.9% (0,25%). The live/dead cell ratio was significantly higher ($p \leq 0.05$) in those embryos derived from the 0.1% group [40.1% ± 23.1%] than that from embryos of the other groups [22.6% ± 13.5% (0%), 23.4% ± 10.4% (0.05%), 24.2% ± 14.6% (0.15%), 22.7% ± 14.0% (0.2%), and 20.3% ± 9.9% (0.25%)]. Apoptotic cells in embryos exposed with 0.1 % and 0.2% DMSO were significantly lower than in those of other groups and with 0.05% DMSO the apoptotic cells in this group are also slightly lower compared to those of control group ($p = 0.08$). Apoptotic index was lower in embryos out of the groups supplemented with 0.1% and 0.2 % DMSO compared to those of the control group (0% DMSO: 3.8%±1.6%, 0.05% DMSO: 2.6%±1.6%, 0.1% DMSO: 2.3%±1.8%, 0.15% DMSO: 3.2%±1.5%, 0.2% DMSO: 2.2%±1.5%, 0.25% DMSO: 3.1%±1.7% [$p = 0.09$; $p = 0.06$]). Fat accumulation was significant higher [$p \leq 0.05$] in embryos stemming from the group supplemented with 0.15 % DMSO (0% DMSO: 6616.9 μm^2±2703 μm^2, 0.05% DMSO: 7346.3 μm^2±1981.3 μm^2, 0.1% DMSO: 6975.5 μm^2±1847.9 μm^2, 0.15% DMSO: 9301.1 μm^2±1703.3 μm^2, 0.2% DMSO: 8675.1 μm^2±2271.4 μm^2, 0.25% DMSO: 8300.7 μm^2±2711μm^2). These findings show that DMSO concentrations of 0.1% and 0.2 % used during in vitro culture influences the quality of embryos at the morphological level. However, further analyses to verify these results at the molecular level via RT-qPCR are still needed.

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