Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS, OOGENESIS, AND SUPEROVULATION

Recombinant FSH (bscrFSH) vs. pituitary FSH (FSH-p): Ovarian response and in vitro embryo production in superovulated Brahman cattle

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Keywords: recombinant FSH, pituitary FSH, superovulation

The use of recombinant FSH is not frequent in *Bos indicus* cattle superovulation (SOV) protocols. The goal of the present study was to determine potential differences in ovarian response and *in vitro* embryo production between a single chain recombinant FSH (bscrFSH) SOV and pituitary FSH (FSH-p) SOV-derived protocol in Brahman cattle. Twenty healthy Brahman cows (*Bos indicus*; body condition score: 3.0-4.5) were randomly divided into two experimental groups (G): G1 (FSH-p: FSH from purified pig pituitary extract; n = 10) and G2 (bscrFSH: recombinant FSH; n = 10). All cows underwent both superovulation treatments in a cross-over design. Regarding G1 SOV, a conventional protocol was applied (Day 0: intravaginal progesterone (P4) device (CIDR: 0.5 g) + 2.0 mg intramuscular (IM) estradiol benzoate E2B + 50 mg P4 (IM); Day 4: total dose = 200 mg of FSH-p divided in 4 day/12 h intervals/8 decreasing doses: 40/40 + 30/30 + 20/20 + 10/10 mg; Day 6: fifth and sixth FSH-p dose + two PGF2α i.m. doses (500 µg of D-cloprostenol each); Day 7: CIDR removal at the seventh FSH-p dose application); Day 8: estrus detection + 1st AI; Day 9: 2nd AI (semen from the same sire was used for all AIs); Day 15 (embryo collection). For G2 cows, the same protocol was applied with modifications (total dose = 195 µg of bscrFSH divided in 4 day/24 h intervals/4 decreasing doses: 40/40 + 30/30 + 20/20 + 7.5/7.5 µg). Ovarian structures [No. follicles (FL on day 8): FL, No. corpora lutea (CL on day 15), and Non-ovulated follicles (NOFL on day 15)] were monitored by using ultrasonography. Moreover, the number of unfertilized oocytes (UFOs) and the number of viable embryos (E) were assessed according to the IETS guidelines. The data were analyzed by GLMM (SPSS® 25, IBM Corp.). No significant differences were detected regarding FL (13.5±1.7 vs. 11.2±2.1) and CL (9.3±1.4 vs. 6.8±1.3) in G1 and G2, respectively ($P > 0.05$). Significant differences were observed in NOFL between G1 and G2 (1.1±0.3 vs. 5.4±2.4; $P \leq 0.05$). No significant differences were observed between G1 and G2 regarding UFOs (1.0 ± 0.4 vs. 0.6 ± 0.3; $P>0.05$); however, significant differences were observed regarding the number of viable embryos (6.9 ± 1.2 vs. 4.2 ± 0.5; $P = 0.05$) when FSH-p and bscrFSH-derived SOV protocols were compared. In conclusion, no differences were observed in the ovarian structures irrespective of the SOV protocol used except in the number of NOFL. The number of viable embryos was greater using the FSH-p-derived SOV protocol. The significant increase in the number of NOFL observed in the bscrFSH SOV protocol could be related to an overstimulation-derived effect at the dose used. Therefore, bscrFSH-dose adjustments will be necessary to apply it successfully in *Bos indicus* cattle.

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FOLLICULOGENESIS, OOGENESIS, AND SUPEROVULATION

Uptake and effects of nanoplastics in maturing oocytes

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Keywords: nanoplastics, oocyte maturation, bovine

Micro- and nanoplastics (MNPs) have raised increasing concerns due to their widespread presence in the environment. The detection of plastic particles in human blood implies that MNPs are circulating in our body and are able to reach organs, including the reproductive tract (Leslie et al., Environ Int, 163:107199, 2022). However, the impact of nanoplastics (NPs) on oocyte development in mammals remains largely unknown, as the focus has mostly been on aquatic animals. In this study, the uptake and impact of NPs in oocytes was investigated with a bovine cumulus-oocyte complex (COC) model. This model is superior to study human reproduction compared to other models, due to the large similarities between bovine and human reproduction during oocyte and embryo development (Sirard, In: Animal Models and Human Reproduction, 127-144, 2017). To determine the uptake of NPs by the cumulus cells and oocyte, bovine COCs collected from slaughterhouse ovaries were in vivo matured (IVM) in medium (NaHCO3-buffered M199 supplemented, with 100 IU/ml Penicillin-streptomycin, 0.05 IU/mL FSH, 0.1 µM cysteamine, and 10 ng/mL EGF) containing 10 µg/mL of 50 nm or 200 nm fluorescently labeled pristine polystyrene (PS) NPs (CD Bioparticles, New York, USA; Polysciences Europe GmbH, Hirschberg an der Bergstrasse, Germany) for 23 h at 39°C and 5% CO2 in air. The dose-response effect on oocyte maturation was studied by exposing COCs during IVM to 50 nm or 200 nm non-fluorescently labeled pristine PS NPs (Polysciences Europe GmbH) at the concentrations of 0, 0.3, 1, 3, 10, and 30 µg/mL. After IVM the uptake and oocyte nuclear maturation stage, defined by the metaphase-II stage of meiosis, was examined via Olympus IXplore SpinSR. One-way ANOVA followed by Holm-Sidak multiple comparisons test was used for statistical analysis. A p-value < 0.05 was considered statistically significant. Confocal microscopy showed that 200 nm NPs were only taken up by cumulus cells, while the 50 nm NPs entered both cumulus cells and oocytes (≥ 80 COCs were imaged per group). In total, 1426 and 1468 COCs in ≥ 5 replicates were analyzed for maturation rate in response to 50 nm and 200 nm NPs respectively. After exposure to 50 nm NPs, there was a significant decrease in oocyte nuclear maturation rate at 3 µg/mL (66.1%) compared to the control group (81.0%, P=0.01). Exposure to 200 nm NPs during COC maturation was not affecting the oocyte nuclear maturation rate at any of the tested concentrations. In conclusion, exposure to 200 nm PS NPs during COC maturation resulted in uptake by cumulus cells, but did not affect oocyte nuclear maturation. Exposure to 50 nm PS NPs resulted in uptake by cumulus cells and oocytes and hampered oocyte maturation at a concentration of 3 µg/mL. The mechanism of 50 nm NPs affecting oocyte maturation needs to be further investigated. Future research will also focus on the endpoints of the oocyte including the competence to develop into an embryo, to further study the impact of NPs exposures on oocyte developmental competence.

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FOLLICULOGENESIS, OOGENESIS, AND SUPEROVULATION

Metabolic stress during the postpartum period in dairy cows might influence follicular growth dynamics at the time of breeding. A retrospective analysis.

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Keywords: negative energy balance, folliculogenesis, fertility

Metabolic and oxidative stress during the early postpartum period is strongly linked with reduced fertility in high-yielding dairy cows. A recent study in our laboratory aimed to examine the associations between blood non-esterified fatty acids (NEFAs) and antioxidant concentrations at week 2 and 8 postpartum (pp) with the granulosa cell (GC) transcriptome in the preovulatory follicles at the time of breeding (at wk 8 pp). For that, estrous synchronization was performed at wk8 by inserting a progesterone releasing intravaginal device (PRID DELTA) for 7 days, and injecting Dinoprost at day 6. Ultrasound-guided transvaginal follicular aspiration of the (largest) dominant follicle (from 27 cows) was performed exactly at 38h after the PRID removal to collect follicular fluid (FF) and granulosa cells as described in Marei *et al.* 2022, JDS 105(8): 6956-6972. The transcriptomic profile of the granulosa cells was determined using RNA sequencing. Based on the expression level of LH surge-responsive genes (*AREG, PTGS2,* SRGN, FST, INHBA, HSPH1, NSDHL, TBC1D8B, PCBD1; Gilbert et al. 2011 Reproduction 141:193-205) we found that 11 cows were already in the post-LH surge phase, while 16 cows were still in the pre-LH surge phase. A significantly higher mean estradiol: progesterone (E2:P4) ratio was confirmed in the FF of the pre-LH compared with the post-LH cohort ($P<0.05$). The aim of the present study was now to perform a retrospective analysis to investigate if the difference in follicular growth dynamics or the timing of LH surge was dependent on the metabolic health of the cow during the early pp period (at wk 2) or at the time of sample collection (at wk 8). We compared wk 2 and wk 8 blood concentrations of NEFAs, antioxidants (β-Carotene, Vitamin E, Glutathione peroxidase, and oxidative stress index), as well as Vit A, Glucose, and Insulin-like growth factor 1. This was done using independent sample T-tests after checking the data homogeneity of variance. None of the analysed wk 2 nor wk 8 blood parameters were different between the two groups, except w2 blood NEFA concentrations, which were significantly higher in the cows that were still in the pre-LH surge phase at the time of sample collection compared with the post-LH cows (mean ± SD: 623±259 vs 411±127 µmol/L, $P<0.05$). This suggests that an elevated blood NEFA concentration at w2 may be associated with a slower follicular development after estrous synchronisation at the time of breeding. Blood and FF NEFA concentrations at wk 8 were not different between the two groups ($P>0.1$), showing that the observed association could be a persistent long-term effect of elevated NEFAs on follicular cell viability or on response to hormonal regulation. While these data may further elucidate why metabolic stress during transition in dairy cows is associated with reduced fertility, further confirmation on a larger scale is needed.

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FOLLICULOGENESIS, OOGENESIS, AND SUPEROVULATION

Beneficial effects of melatonin in hypoxic condition on canine oocyte nuclear maturation through reduction of oxidative stress

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Keywords: Oocyte, Melatonin, Hypoxia

Unlike other domestic animals, in vitro maturation of oocytes in canine results in very low nuclear maturation rate accompanied with high degeneration. High fat content of canine oocytes predisposes them to oxidative stress and production of high levels reactive oxygen species (ROS). Melatonin (MTN) a highly lipophilic hormone with a strong antioxidant effect which acts through its G- protein coupled receptors (MTNR-A1and B1) has been proven beneficial in supporting oocytes during culture.

Canine cumulus oocyte complexes (COCs) were collected after routine ovariohysterectomy and processed by chopping the ovarian cortex, for the following experiments. Each experiment was replicated four times.

Experiment 1: analyzed expression of MTNRs by immunofluorescence staining. MTNR-1 A and 1 B were highly expressed in the oocytes and with lower intensity in the cumulus cells. The distribution of MTNR-A1and B1 signal didn't follow a nuclear or peri-nuclear pattern and both were evenly scattered within the ooplasm.

Experiment 2: The COCs (n=300) with three or more layers of cumulus cells, were cultured in the absence (control) or presence of 100nM, for 72h in two groups; [1] low O2 (5%) and [2] high (20%) in air at 38.5ºC. Nuclear stage of the oocytes in meiosis was determined under Nikon fluorescent microscope, after denudation and fixation and Hoechst staining. Melatonin at 100nM concentration had a beneficial effect on the nuclear maturation profile of canine oocytes in both conditions. However, the oocytes cultured in low O2 versus cultured in high O2 for 72h exhibited the lowest percentage of oocytes at GV stage (6.7%±4.2 vs 19.8%±3), highest MII maturation rate (32.3%±6.4 vs 15.81%±8.1), minimum degeneration (20.5% ± 3.2 vs 45.2%±5.15) and maximal meiotic resumption (GVBD-MII; 56.2%±8.6 vs 19%±3), when the basic maturation medium was supplemented with 100nM of melatonin in ($P < 0.05$).

Experiment 3: analyzed the effects of melatonin on production of ROS using DCHFDA staining. Densitometry using ImageJ software showed that the overall intensity of fluorescence was lower in oocytes treated with 100nM melatonin ($p<0.05$).

Experiment 4: analyzed impact of melatonin supplementation on expression level of genes related to ROS repairing enzymes (GPX1, SOD1, SOD2, GSR & CAT). Freshly collected and in vitro matured COCs were snap-frozen (25 COCs per group/repeat) after in vitro maturation and used for RNA extraction and qRTPCR. Melatonin supplementation in high O2 reduced the expression of all ROS repairing enzymes significantly. It also reduced the GPX-1 ($p<0.005$), catalase ($P<0.01$) expression in the COCs cultured in low O2.

Overall, these data suggest that melatonin protection of oocytes from oxidative stress results in reduced degeneration and increased nuclear maturation. The beneficial effect of melatonin supplementation during in vitro maturation of dog oocytes in production of developmentally competent oocytes requires further investigation.

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FOLLICULOGENESIS, OOGENESIS, AND SUPEROVULATION

Endocrine disrupting chemicals induce alterations in lipid droplets of bovine cumulus cells

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Keywords: endocrine disrupting chemicals, cumulus cells, lipid droplets

Endocrine disrupting chemicals (EDC), omnipresent in the human environment, are potentially harmful to female fertility (Panagopoulos P. et al., Best Pract Res Clin Obstet Gynaecol, 2023). We developed a test strategy for effects of EDCs on female fertility by using the relevant animal model of bovine oocyte in vitro maturation (IVM), which complies with the 3Rs. To identify endpoints sensitive to EDC activity, biological processes within the cumulus oocyte complex (COC) were previously tested upon exposure to known EDCs. Here, we report the effect of EDCs ketoconazole (KTZ; CYP450 enzyme inhibitor) and diethylstilbestrol (DES; estrogen receptor agonist) on lipid droplet (LD) dynamics, considering the importance of lipids in oocyte developmental competence. COCs were matured for 24h (as reported in Asimaki K. et al., Front. Toxicol., 2022) either with vehicle DMSO (0.01% v/v for DES, 0.1% v/v for KTZ), KTZ (10^{-8} M, 10^{-7} M, 10^{-6} M) or DES (10^{-9} M, 10^{-7} M, 10^{-5} M) (Sigma-Aldrich, Missouri, USA). COCs (45 per group) were stained with the specific neutral lipid dye LD540 (courtesy of Spandl J., Traffic., 2009) and cumulus cells (CCs; over 20000 per group) were imaged with confocal microscopy (top-to-bottom, 1μm z-step). LD count per cell, area, and clustering (% of LDs within a cluster) were recorded and analysed with IBM SPSS statistics software to compare groups with one-way ANOVA, followed by Tukey's post hoc test. KTZ treatment resulted in a concentration-independent increase of LD area in CCs at all concentrations (increasing KTZ: 0.40±0.04, 0.40±0.05, 0.41±0.04 μm^2; vs vehicle control 0.37±0.04 μm^2, $p < 0.001$). In addition, LDs were more clustered (70±9%, 66±13%, 73±8%; increasing KTZ) compared to the vehicle-control (56±16%, $p<0.001$). The LD count per cell was comparable between the vehicle-control (9±2) and KTZ-treated groups (9±2, 9±2, 9±2; increasing KTZ). DES treatment of COCs led to an increase of LD area in CCs exposed to 10^{-9} M (0.44±0.15 μm^2) and 10^{-5} M (0.44±0.04 μm^2) DES, compared to the vehicle group (0.39±0.03 μm^2, $p<0.001$), but no such effect was observed at 10^{-7} M (0.4±0.05 μm^2) DES. CCs exposed to 10^{-7} M DES had a slight increase in LD clustering (66±13%) compared to the vehicle group (61±12%, $p<0.005$). Finally, LD count slightly decreased at 10^{-5} M DES (7±2%) when compared to vehicle- and DES-groups (9±2%, $p<0.001$). We have previously shown that KTZ (unpublished) or DES do not affect cell viability but 10^{-8} M KTZ reduces blastocyst rate and 10^{-5} M DES halts oocyte nuclear maturation. Here we report that KTZ treatment possibly resulted in an increase in lipid content, based on the larger LD area, as well as LD clustering, in a concentration-independent manner. DES treatment at all concentrations affected the LD organization dynamics. LD area was increased at 10^{-9} M DES, suggesting a potential increase in lipid content. A similar effect was observed at 10^{-5} M DES, concurrent with a reduction in LD count per cell. Finally, LD clustering was increased only at 10^{-7} M DES. In the future, these observations will be validated by lipidomic analysis of the neutral lipid content of cumulus cells.

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