Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Folliculogenesis, oogenesis, and superovulation

Superovulation efficiency and embryo production by using recombinant FSH (bscrFSH) vs. pituitary-derived FSH (FSH-p) in Brangus heifers

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During the last decades the use of the conventional pituitary-derived FSH in superovulation (SOV) protocols have been associated with low yield and quality of the obtained embryos. The objective of the present study was to determine whether the stimulation with a single dose of bovine long-acting recombinant FSH (bscrFSH) could achieve an efficient SOV response compared to the conventional pituitary-derived FSH (FSH-p) in Brangus cattle. Sixteen healthy Brangus heifers (BW: 450± 50 kg; BCS: 3.5± 0.5) were randomly distributed into two groups: conventional [FSH-p: FSH from Purified Pig Pituitary Extract (Pluset, Buenos Aires, Argentina); n= 8] vs. recombinant group [bscrFSH: recombinant FSH (Cebitropin B, Concepción, Chile); n= 8]. The conventional SOV protocol was applied as follows: Day 0: intravaginal P4 device (CIDR: 1.38 gr) + 2.5 mg (i.m.) 17-β Estradiol + 50 mg P4 (i.m.); Day 4: 330 IU FSH-p/12 h intervals/4 d/8 decreasing doses, 60-60, 50-50, 35-35, 20-20, in total 8 injections; Day 6: 5th and 6th FSH-p dose + two PGF2α i.m. doses (500 µg D-cloprostenol each); Day 7: CIDR removal (p.m) at the 8th FSH-p dose application; Day 9: 0.0105 mg (i.m.) Buserelin Acetate (a.m) + AI (p.m.); Day 10: AI (a.m.). Regarding the recombinant group, the same protocol was applied with modifications (150 µg bscrFSH/24 h intervals/4 d/4 decreasing doses, 55-45-30-20, in total 4 injections). Ovarian structures [follicles (FL), corpora lutea (CL), and non-ovulated follicles (NOF)] were monitored by using ultrasonography on Day 8 (estrus; FL number) and Day 15 (embryo collection; CL and NOF number). Morphological embryo classification and quality were performed according to the IETS guidelines. The data were analysed by GLMM (SPSS® 25, USA). No statistical differences were observed between SOV protocols regarding FL, CL, or NOF ($p > 0.05$). Significant differences were observed in the number of total structures collected (15.8±2.9 vs. 6.0±1.3; bscrFSH-derived vs. FSH-p-derived SOV protocol, respectively; $p= 0.01$). Although no significant differences were observed in the number of non-transferable embryos (3.5±0.8 vs. 1.6±0.4; $p=0.07$), significant differences were observed in the number of non-fertilised oocytes (UFOs: 1.8±0.6 vs. 0.5±0.2; $p=0.04$) as well as in the number of degenerated embryos obtained (DE: 1.5±0.5 vs. 1.0±0.3; $p= 0.02$). However, no statistical differences were detected in the number of viable embryos obtained (9.2±2.6 vs. 4.38±1.2) when bscrFSH and FSH-p-derived protocols were compared ($p= 0.08$). In conclusion, the number of total structures, UFOs, and DE increased substantially per donor by using the bscrFSH-derived SOV protocol. Despite of the increase in the number of viable embryos obtained in Brangus heifers by using the bscrFSH-derived SOV protocol no differences were observed between groups. ANID 21201280.

Keywords: recombinant FSH, embryo production, heifers

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Prolonged application of recombinant FSH (bscrFSH) in superovulation protocols: in vivo embryo production in Bos taurus cows in tropical environments

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Bovine recombinant follicle-stimulating hormone (bscrFSH) has been used very occasionally in *Bos taurus* superovulation (SOV) protocols. The main aim was to study the effects of prolonged bscrFSH application in SOV protocols to test the differential effects on *in vivo* embryo production in *Bos taurus* cows under tropical environments. A total of 10 healthy Charolais cows (age: ~60 mo.; BW: ~750±50 kg; BCS: 3.75-4.0) located in Morona-Santiago province, Ecuador (Köppen-Geiger: Af; Precip.:~1,200 mm; R.H.:~92%; M.T.:~22 °C latitude: 2°18'22.41"S / longitude: 78°6'55.34"W; altitude: ~1,020 m.a.s.l.) were divided randomly into 2 groups (G1: 4-day bscrFSH (Cebitropin B, Concepción, Chile), and G2: 5-day bscrFSH application; n=5 each). The G1 SOV protocol was applied as follows: Day 0: intravaginal P4 device (CIDR: 1.38 gr) + 2.5 mg intramuscular (i.m.) Estradiol Benzoate E2B + 100 mg P4 (i.m.); Day 4: 180 µg bscrFSH-r/24 h intervals/4 d/4 decreasing doses; Day 6: 3rd bscrFSH dose + two PGF2α i.m. doses (12 h interval/ 500 µg D-cloprostenol each, am/pm); Day 7: CIDR removal at the 4th bscrFSH dose application; Day 8: 0.02 mg GnRH + AI; Day 15: embryo collection. Regarding G2, the same protocol was applied with modifications: Day 0: same; Day 4: 180 µg bscrFSH-r/24 h intervals/5 d/5 decreasing doses; Day 7: 4th bscrFSH dose + two PGF2α i.m. doses (12 h interval/ 500 µg D-cloprostenol each, am/pm); Day 8: CIDR removal at the 5th bscrFSH dose application; Day 9: 0.02 mg GnRH + AI; Day 16: embryo collection. Ovarian-derived traits scored: number of corpora lutea (NCL) and non-ovulated follicles (NOF). Embryo-derived traits scored: total structures (TS), transferable embryos (TE), morulae (M), early blastocysts (EBL), blastocysts (BL), degenerated embryos (DE), unfertilized oocytes (UFOs), and non-transferable structures (NTS). The data were analysed by GLMM (SPSS® 25, USA). Significant differences were observed in EBL (7.75±2.65 vs. 0.75±0.75; p=0.04) and BL (2.50±1.55 vs. 0.20±0.20; p=0.03) in G2 and G1, respectively (p<0.05). Non-significant differences were detected between G1 and G2 SOV protocols when ovarian-derived traits and several embryo-derived parameters (TS, TE, and DE) were compared (p> 0.05). However, significant differences were observed in UFOs (5.75±2.90 vs. 2.00±0.90 for G1 and G2, respectively; p =0.003) and NTS (7.25±2.92 vs. 3.75±0.75 for G1 and G2, respectively; p =0.015) between protocols, being G2 lower in both parameters. In conclusion, no differences were observed regarding ovarian-derived traits between bscrFSH-derived protocols. The G2 protocol was the most efficient for EBL and BL production together with lower values of UFOs and NTS. These differences may be related to a prolonged ovarian stimulation during the application of the G2 SOV protocol in *Bos taurus* cows under tropical conditions. ANID 21201280.

Keywords: recombinant FSH, embryo production, cows

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Optimal blood anti-oxidant concentrations at the time of breeding may enhance preovulatory granulosa cell functions after negative energy balance in dairy cows. A transcriptomic insight.

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Early postpartum metabolic stress in high-yielding dairy cow s is strongly linked with reduced fertility. We have recently demonstrated that negative energy balance (NEB) and the increase in non-esterified fatty acids (NEFAs) at week 2 postpartum (pp) are associated with long-term changes in the granulosa cell (GC) transcriptome in the preovulatory follicles at the time of breeding (w8 pp) (Marei et al. 2022, *JDS*). Genes involved in cellular stress and inflammatory responses, and responses to lipids and ketones were upregulated in the GCs of NEB cows compared to those with basal blood NEFAs at w2. In the present study we aimed to evaluate the potential interaction between blood antioxidants (AO) and NEFAs on GC functions. We hypothesized that an optimal AO status may attenuate the long-term effects of NEFAs on the ovarian follicle.

To test this hypothesis, we used our RNAseq database of w8 preovulatory (pre-LH surge) follicle GCs collected from cows with known w2 and w8 blood AOs (βCarotene, βC; Vitamin E, VitE) and NEFA concentrations (n=16). For this study, we selected the cows with above median w2 blood NEFAs (0.78±0.19 mM, n=10). These cows were then split into 2 subgroups (n= 4-6) based on median values of blood βC and VitE, either at w2 (High AOs: 3.0±0.9 mg/dL βC and 2.9±0.4 mg/dL VitE *vs.* Low AOs: 1.3±0.3 mg/dL βC and 1.7±0.3 mg/dL VitE) or at w8 (High AOs: 5.5±1.9 mg/dL βC and 6.5±1.4 mg/dL VitE *vs.* Low AOs: 2.3±0.6 mg/dL βC. and 3.0±1.0 mg/dL VitE). The GC transcriptomic profiles of these subgroups were compared using a DESeq2 analysis at each timepoint to determine the differentially expressed genes (DEGs: *P*-adj<0.05, 5% FDR). DEGs were functionally annotated using Bioconductor packages in R.

Only 3 DEGs (3↑, 0↓) could be detected in the w2 comparison, whereas 194 DEGs (48↑, 146↓) were detected in the w8 comparison. The enriched upregulated pathways of the w8 comparison are related to activation of meiosis, MAPK signaling, IGF and EGF receptor signaling, as well as genes involved in fertilization. These are indicators of a better oocyte supportive capacity. High AOs also appear to increase active RNA biosynthetic processes, amino acid and carbohydrate metabolism, and mitotic activity (cell proliferation) as indicated by the upregulated pathways, suggesting better cell viability and follicle quality. The downregulated pathways indicate lower levels of inflammation and cellular stress because genes related to mitochondrial fragmentation, DNA breakdown, sphingomyelin biosynthesis and apoptosis were downregulated.

In conclusion, these results strongly suggest that GCs from follicles exposed to elevated NEFA during their early growth phases exhibit reduced cell stress levels and lower oxidative damage when the final follicle preovulatory development takes place under optimal antioxidant concentrations. In other words, a high blood antioxidant profile in dairy cows at the time of breeding may alleviate, at least in part, the impact of NEB on GC functions.

Keywords: antioxidants, granulosa cell transcriptome, metabolic stress

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Effects of the endocrine disruptor ketoconazole on bovine oocyte maturation and blastocyst development

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Endocrine disrupting chemicals (EDCs) can negatively affect the reproductive system, as evidenced by studies on animal models and human cell lines (Ratan S. et al., J. Endocrinol, 233(3), 2017). Exposure to such chemicals may cause detrimental effects on female reproductive health, increasing the need to improve regulatory reproductive toxicity assessment (van Duursen M. et al., Int. J. Mol. Sci, 21(3215), 2020). Broadening of the methods currently adopted, for example to include assessment of effects on oocyte maturation and developmental competence acquisition, is needed. Here, we present an assay identifying EDCs eliciting reproductive toxicity based on a bovine model of *in vitro* oocyte maturation and embryo production. To show the applicability of the assay, the known human-relevant endocrine disruptor ketoconazole (KTZ; a CYP450 inhibitor) was used. Endpoints explored include nuclear maturation, cumulus cell expansion, steroidogenesis and blastocyst rate.

Immature cumulus-oocyte complexes (COC) were isolated from ovaries excised from slaughterhouse cows post-mortem. The COCs were *in vitro* matured for 24h in defined culture media, as previously described (Brinkhof B. et al., BMC Gen., 16(1), 2015), and supplemented with KTZ (10^{-8} M, 10^{-7} M, 10^{-6} M; Sigma-Aldrich, Missouri, USA) diluted in DMSO (vehicle; Sigma-Aldrich, Missouri, USA). Oocytes were stained with DAPI to evaluate nuclear maturation based on the presence of an MII plate and a polar body. KTZ- and vehicle-treated oocytes had comparable nuclear maturation rates (ascending KTZ M; 81%±3 ,76%±3, 65%±15 vs 84±1 vehicle-treated, n=486). Similarly, KTZ did not affect cumulus cell expansion (n=725); measured as fold-increase of the projected COC surface and calculated from images acquired pre- and post-IVM. To further investigate cumulus cell function, steroid hormone secretion into the media was quantified by LC/MS. Progesterone, estrone, and 17β-estradiol were present in levels above the detection limit. Progesterone secretion by COCs exposed to 10^{-6} M KTZ was reduced by 88% (733±168 pg/ml vs 6326±1169 pg/ml, one-way ANOVA, $p < 0.001$, n=1050), not observed at lower KTZ doses. To determine effects on developmental competence, exposed COCs were used for IVF and zygotes were cultured *in vitro* (IVC) for 8 days without KTZ. Oocyte exposure to 10^{-8} M KTZ resulted in a reduction of the D8 blastocyst rate (21%±5 vs 31%±3, n=1246, one-way ANOVA, $p < 0.05$), not observed at higher KTZ doses. To assess impact on early embryo development, zygotes produced from non-exposed oocytes were exposed to KTZ in IVC. No effect on D8 blastocyst rate was observed at any KTZ dose (n=991). At least three biological replicates were performed for all endpoints.

In conclusion, exposure of COCs to KTZ impacted developmental competence and steroidogenesis. Oocytes were more sensitive to KTZ than cumulus cells, while embryos were not sensitive to KTZ. In the future, we will be expanding this assay to include more endpoints (e.g. cytoplasmic maturation, blastocyst quality) and verify the validity of the model for a range of EDCs with distinct action.

Keywords: endocrine disruptor, oocyte, bovine

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Folliculogenesis, oogenesis, and superovulation

Optimization of a superovulation protocol for the collection of in vivo matured oocytes from Holstein Friesian heifers

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In vivo matured oocytes have superior developmental competence to those matured *in vitro*. As such, the study of *in vivo* oocyte maturation supports the refinement of the *in vitro* maturation system. Therefore, this work aimed to optimize a protocol to collect *in vivo* matured oocytes from Holstein Friesian heifers. To do so, animals (n=5) were synchronized using an intravaginal device (CIDR, Zoetis Belgium). Eight days later, the device was removed and heifers received 0.5 mg of cloprostenol (2 ml of Cyclix (prostaglandin; PG) Virbac, France). Estrus (=day 0) was confirmed two days later by ultrasound. To induce ovulation, the heifers were treated with 10 µg of buserelin (2.5 ml of Receptal (GnRH), Intervet, Germany) on day 8 of the estrous cycle. From day 10, the heifers were superovulated with 180 mg of FSH (Folltropin, Vetoquinol, Canada) administered twice daily for four days in decreasing doses (1.5, 1.5, 1.25, 1.25, 1, 1, 0.75 and 0.75 ml respectively). On day 12, animals received 0.75 mg (3 ml) of PG and LH surge was induced with 10 µg (2.5 ml) GnRH administered 40 h after the PG injection. To determine the optimum timing of follicle aspiration, we tested four timing protocols (P1, P2, P3, P4) based on the time-post-GnRH treatment (P1=20h, P2=21.5h, P3=22.5h and P4=24h) at which OPU was performed. Only one animal was used in each protocol, except in P4 which included two heifers. Follicles ≥ 8mm in diameter were aspirated using a 18 gauge needle. A 130 mmHg vacuum pressure was used due to the presence of expanded and sticky cumulus cells characteristic of oocyte maturation. Before follicle aspiration, the OPU tubing system was rinsed with PVP medium (0.3% PVP (PVP-360; Sigma) in Ca- and Mg-free PBS + 10 IU/ml heparin (Sigma)). The follicular content was collected in a 50 ml conical tube and cumulus-oocyte complexes were recovered under a stereomicroscope and stored in EmXcell medium without BSA (imv-technologies, France) until the end of the collection procedure. To assess maturation status, all oocytes (n=45) were denuded in 0.1% hyaluronidase (Sigma) in PVP medium for approximately 3 min and by pipetting until all cumulus cells were removed from the oocyte. Subsequently, the presence of the first polar body (PBI) was evaluated and the denuded oocytes were stored for further molecular analysis. In P1, a total of 8 oocytes were collected and the PBI-extrusion rate was 0%. Similarly, in P2, we collected 3 oocytes and the PBI-extrusion rate was 0%. Interestingly, at 22.5h after GnRH injection (P3), the PBI extrusion rate was 20% (2/10). A greater PBI extrusion rate was observed in P4 where the rate was 37.5% for both heifers (3/8 and 6/16) used in this protocol. The present results suggest that the optimum timing of aspiration of *in vivo* matured oocytes is beyond 24h after GnRH injection. Moreover, the hormonal stimulation treatment we used could be considered an efficient method to collect in vivo matured bovine oocytes. This work also provides insights into using defined and serum-free media when performing non-standard experiments on livestock for further molecular studies on *in vivo* oocyte maturation.

Keywords: OPU, oocyte, cattle