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Influence of choline and follistatin supplementation during in vitro bovine oocyte maturation on oocyte competence and blastocyst development

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Research Article

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Summary

Metabolite supplementation during *in vitro* embryo development improves blastocyst quality, however, our understanding of the incorporation of metabolites during *in vitro* maturation (IVM) is limited. Two important metabolites, follistatin and choline, have beneficial impacts during *in vitro* culture; however, effects of supplementation during IVM are unknown. The objective of this study was to investigate combining choline and follistatin during IVM on bovine oocytes and subsequent early embryonic development. We hypothesized that supplementation of choline with follistatin would synergistically improve oocyte quality and subsequent early embryonic development. Small follicles were aspirated from slaughterhouse ovaries to obtain cumulus oocyte complexes for IVM with choline (0, 1.3 or 1.8 mM) and follistatin (0 or 10 ng/mL) supplementation in a 3 × 2 design. A subset of oocytes underwent transcriptomic analysis, the remaining oocytes were used for IVF and *in vitro* culture (IVC). Transcript abundance of *CEPT1* tended to be reduced in oocytes supplemented with 1.8 mM choline and follistatin compared to control oocytes ($P = 0.07$). Combination of follistatin with 1.8 mM choline supplementation during maturation, tended ($P = 0.08$) to reduce *CPEB4* in oocytes. In the blastocysts, *HDCA8*, *NANOG*, *SAV1* and *SOX2* were increased with choline 1.8 mM supplementation without follistatin ($P < 0.05$), while *HDCA8* and *SOX2* were increased when follistatin was incorporated ($P < 0.05$). The combination of choline and follistatin during oocyte maturation may provide a beneficial impact on early embryonic development. Further research is warranted to investigate the interaction between these two metabolites during early embryonic development and long-term influence on fetal development.

Introduction

One of the important aspects of producing *in vitro* embryos is oocyte quality following *in vitro* maturation (IVM) (Gilchrist and Thompson, 2007). The process of IVM requires aspiration of immature oocytes from small antral follicles and their incubation in an environment mimicking *in vivo* maturation. Research to improve the composition of maturation medium has focused on different hormonal additions to aid the oocyte to complete maturation (Izadyar *et al*., 1998; Sutton-McDowall *et al*., 2012). While hormone supplementation is important during IVM, nutritional metabolites provide a beneficial impact on oocyte quality and subsequent embryonic development (Ferguson and Leese, 2006; Warzych and Lipinska, 2020). Follistatin beneficially impacted embryonic development when supplemented during culture (Silva and Knight, 1998). Follistatin is a secreted glycoprotein that inhibits follicle stimulating hormone (FSH) production and can target TGFβsuperfamily members to regulate cellular metabolism (Zhenhua *et al*., 2017). Other nutrients supplemented during *in vitro* embryo culture improved embryo development by altering DNA methylation (Ali and Sirard, 2002; Estrada-Cortes *et al*., 2021; Knitlova *et al*., 2017). Among the nutrients supplemented through IVP, choline demonstrates promising influence on blastocyst development.

Choline is a one-carbon metabolite that is important during cellular methylation, formation of acetylcholine and biosynthesis of phosphatidylcholine (Estrada-Cortes *et al*., 2020). Supplementation of rumen-protected choline in dairy cows did not influence overall reproductive performance (days to first ovulation, dominant follicle growth or diameter), but down-regulated transcript abundance of pro-inflammatory markers (Acosta *et al*., 2017). In a recent study by Estrada-Cortes, supplementation of choline at 1.3 mM during IVP altered global DNA methylation at the blastocyst stage (Estrada-Cortes *et al*., 2020). Embryos produced *in vitro* with supplementation of choline 1.8 mM and subsequently transferred into recipient cows resulted in calves with improved birth weights and weaning weights (Estrada-Cortes *et al*.,

2021). Addition of choline during IVP may have a positive impact on the early embryo. Choline supplementation during oocyte maturation has not been investigated.

Providing an optimum environment is crucial for setting up the oocyte for greater success during fertilization and subsequent early embryonic development. Therefore, we hypothesize that supplementing a combination of follistatin and choline during IVM will improve oocyte quality and subsequent early embryonic development *in vitro*. The overall objective of this study was to determine the effect of combining follistatin and choline supplementation during IVM on oocyte quality and early embryonic development.

Material and methods

Experimental design

Cumulus oocyte complexes (n = 1,600/treatment) were matured with basal IVM medium or supplementation: two concentrations (1.3 and 1.8 mM) of choline chloride, 10 mg/mL follistatin or a combination of choline chloride and follistatin in a 3 × 2 factorial design. Choline concentrations were selected based on research performed by Estrada-Cortes *et al.* (2020), using the two concentrations that demonstrated an impact during early embryonic development. Concentration of follistatin was selected based on research performed by Zhenhua *et al.* (2017). Follistatin was solubilized in phosphate-buffered saline (PBS) with 0.1% BSA. To ensure similar basal media across treatment groups, PBS with 0.1% BSA was added into control, choline 1.3 mM and choline 1.8 mM IVM media. Eight replicates were completed with 200 COCs randomly assigned to a treatment and cultured in different IVM media conditions (control, follistatin, choline 1.3 mM, choline 1.8 mM, choline 1.3 mM × follistatin and choline 1.8 mM × follistatin).

After IVM, cumulus cell expansion was visually graded non-blinded based on Smits *et al.* (2020) with modifications (excellent/good: fully expanded cumulus cells with three or more layers; fair: moderate expansion of cumulus cells with three or more layers; poor: no expansion of cumulus cells or loss of cumulus cells) to determine the influence of treatments. A subset of COCs (n = 800/treatment) were stripped of cumulus cells by vortexing in HEPES medium with hyaluronic acid (1,000 units/ml μg) and pools of 40 oocytes were flash frozen in liquid nitrogen and stored at −80°C until RNA extraction and analysis for transcript abundance. Cumulus cells were pelleted from 50 oocytes, flash frozen in liquid nitrogen and stored at −80°C until RNA extraction and analysis for transcript abundance. After IVM, a subset of matured COCs (n = 800/treatment) were fertilized in SOF-FERT medium and after denudation cultured in SOF-BE1 medium. Cleavage rates (number of PZ that completed the first cell division) were recorded on day 2 to determine fertilization rate, further, blastocyst rates were recorded on day 7. A subset of PZ (n = 14/treatment) were placed into a MIRI® timelapse incubator (Esco Medical, Horsham, PA) to determine timing of specific embryonic cell stages. On day 8, approximately 40 blastocysts per treatment group were collected to determine inner cell mass (ICM) and trophectoderm (TE) cell numbers or pooled in groups of 10, flash frozen and stored at −80°C until RNA extraction and analysis for transcript abundance.

Oocyte maturation and embryo production

All chemicals and hormones were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. All media and culture procedures for IVM, *in vitro* fertilization (IVF) and IVP of embryos has been previously described by Snider and colleagues with modifications to the IVM medium for supplementation of choline and follistatin (Snider *et al.*, 2023). Briefly, ovaries from various beef cattle breeds were obtained at a local abattoir and cumulus-oocyte complexes (COC) were recovered by manually aspirating follicles of 2–7 mm in diameter using a 21-g needle and 3 cc syringe. Aspirated follicular fluid was transferred into a searching dish containing oocyte collection medium (OCM; BoviPlus, MiniTube, Verona, WI). Cumulus oocyte complexes grades 1-2 (Thibier and Perry, 2023) were selected (n = 50/well; 2 well/treatment; 4 replicates) and washed with OCM and oocyte maturation medium (OMM) containing cell culture grade water, TCM-199 (Thermo Fisher Scientific, Waltham, MA), 10% adult bovine serum, 26.19 mM NaHCO3, 50 μg/mL gentamicin, 1 mM Glutamax (Thermo Fisher Scientific, Waltham, MA), 25 μg/mL Folltropin-V (Bioniche, Athens, GA), 2 μg/mL estradiol and 0.25 mM sodium-pyruvate. Washed COC were placed into a 4-well dish containing 500 μl OMM and cultured between 22 to 24 hr at 38.5° C in a humidified atmosphere of 5% CO_2. After IVM, matured COC were washed once with HEPES medium containing: 10 mM HEPES, 1.17 mM calcium chloride dihydrate, 0.49 mM magnesium chloride hexahydrate, 1.19 mM potassium phosphate monobasic, 7.16 mM potassium chloride, 2 mM chloride, 2 mM sodium bicarbonate, 107.7 mM sodium chloride 6.6 mM sodium lactate, 0.3% BSA fraction-V, 7.5 μg/mL gentamicin and 0.2 mM sodium-pyruvate and once with fertilization medium (SOF-FERT) containing cell culture grade water, 1.17 mM calcium chloride dihydrate, 0.49 mM magnesium chloride hexahydrate, 1.19 mM potassium chloride, 25.07 mM sodium bicarbonate, 107.7 mM sodium chloride, 6.6 mM sodium lactate, 0.6% BSA fatty acid free, 5 μg/mL gentamicin, 0.2 mM sodium-pyruvate, 2 U/mL heparin and 757 μM caffeine, then transferred into a 4-well plate containing SOF-FERT medium. PureSperm® (Spectrum Technologies, Healdsburg, CA) separation procedure was used on semen from a single sire and diluted with SOF-FERT medium to a final concentration of 1×10^6/mL, 60 μl of diluted sperm was added to each well and incubated for 18–20 hr at 38.5°C in humidified air with 5% CO_2. Presumptive zygotes (PZ) were denuded after fertilization by vortexing in HEPES medium and hyaluronic acid (1,000 U/mL), washed with HEPES and SOF-BE1 medium containing cell-culture grade water, 1.17 mM calcium chloride dihydrate, 0.49 mM magnesium chloride hexahydrate, 1.19 mM potassium phosphate monobasic, 7.16 mM potassium chloride, 25.07 mM sodium bicarbonate, 107.7 mM sodium chloride, 6.6 mM sodium lactate, 0.4% BSA fatty acid free, 25 μg/mL gentamicin, 0.4 mM sodium-4 mM pyruvate, 1x essential amino acids, 1 mM Glutamax (Thermo Fisher Scientific, Waltham, MA), 1x non-essential amino acids, 2.77 mM myo-inositol and 0.6 mM sodium citrate. A subset of PZ (n = 14/treatment) was individually cultured in a Culture Coin® (Esco Technologies, Horsham, PA) and placed into a MIRI® TimeLapse incubator (Esco Technologies, Horsham, PA) to determine timing to specific embryonic developmental stages. The remaining PZs were placed into 30 μl SOF-BE1 drops covered with mineral oil and cultured at 38.5°C with 5% CO_2, 5% O_2 and 90% N_2 in humidified air from day 1 to day 8. Cleavage rate was evaluated on day 3 and blastocyst development was evaluated on day 8 of development.

Transcript abundance

Matured oocytes and cumulus cell pellets were subjected to RNA extraction using the Qiagen RNeasy Micro kit (Qiagen, Valencia,

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CA) following manufacturer's instructions and quality of RNA was determined with a Nanodrop 8000 (ThermoFisher Scientific, Waltham, MA) and an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA). Following extraction, complementary DNA (cDNA) was made using the iScript Advanced cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) following manufacturer's instructions using 80- and 2-ng of total RNA from cumulus cell and oocyte pools, respectively. Following reverse transcription, cDNA was diluted to 250- and 12.5-pg/µl final working concentration for cumulus cell and oocyte pools, respectively.

Blastocysts were subjected to RNA extraction using the Qiagen RNeasy Micro kit (Qiagen, Valencia, CA) following manufacturer's instructions and quality of RNA was determined using the same methods used for the oocytes and cumulus cells. After the extractions were completed, cDNA was made using the iScript Advanced cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) following manufacturer's instructions using 1 ng of total RNA from blastocyst pools. Following reverse transcription, cDNA was diluted to 3.125 pg/µl final working concentration for blastocyst pools.

Real-Time RT PCR was performed for cumulus cell, oocyte and blastocyst cDNA following the protocol listed in Snider *et al.* (2022). Briefly, 2 µl (500 pg, 25 pg and 6.25 pg equivalence cDNA for cumulus cell, oocyte and blastocyst pools, respectively) of cDNA was added to a 20 µl reaction with 10 µl iTaq Universal SYBR Green Mastermix (Bio-Rad Laboratories, Hercules, CA) and 1 µl each of forward and reverse primers (10 µM; primer sets listed in Supp Tables 1–3). Quantification of transcript abundance by RT-PCR was performed on a CFX96 Real-time system (Bio-Rad Laboratories, Hercules, CA) and the endogenous reference gene was glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), which was constituently expressed across all treatments within cell type (data not shown). Intra-assay coefficients of variation for *GAPDH* and the target genes were ≤ 20%. Relative transcript abundance was measured following the comparative CT method (Livak and Schmittgen, 2001). The calibrator was set as the lowest expressing sample for each respective transcript and transcript abundance was expressed as a fold change relative to this calibrator following comparative CT calculation.

Blastocyst cell numbers

ICM and TE cell numbers were determined in a subset of blastocysts as described in Wooldridge and Ealy (2019). Briefly, blastocysts representative of different stages (non-expanded, expanded and hatched) within each treatment group were selected. Blastocysts were washed twice with phosphate-buffered saline and 0.2% polyvinyl pyrrolidone (PBS-PVP) and then fixed with 4% [*w/v*] paraformaldehyde at room temperature for 15 min, washed three times with wash buffer containing 0.1% [*v/v*] Tween20 and 0.1% BSA (WB), permeabilized with 0.25% [*v/v*] Triton-X at room temperature for 20 min and blocked with 10% [*v/v*] donkey serum at room temperature for 1 hr. Blastocysts were then incubated at 4°C overnight with anti-CDX2 (MU392A-5UC, Biogenex, 1:100 dilution), washed three times with WB and incubated at room temperature for 1 hr with donkey-anti-mouse FITC (Invitrogen, A16108, 1:200 dilution) in a dark room. After incubation, blastocysts were washed three times with WB, then blastocyst DNA was stained with DAPI (1 µg/ml) at room temperature for 5 min. Blastocysts were then placed in ProLong™ Gold Antifade and imaged by flattening on a glass slide outlined with a thin layer of petroleum jelly. A Lecia DM6B microscope equipped with a Lecia CTR6 LED illumination system was used to visualize CDX2 and DAPI staining. Images were captured with a Lecia DFC7000T digital camera and assembled with Las X software (Lecia Microsystems, Deerfield, IL). The same program was used to label and record individual nuclei by counting nuclei stained for CDX2 ($CDX2^+/DAPI^-$, indicating TE) and only DAPI ($CDX2^-/DAPI^+$, indicating ICM).

Statistical analysis

This study was performed as a 3 × 2 factorial design with choline (0, 1.3, 1.8 mM), follistatin (0, 10 ng/mL) or the combination of the two different treatments. Percentage data (COC quality, cleaved and blastocyst stage) were analysed using the GLIMMIX procedure with the logit function in SAS (SAS Inst. Inc., Cary, NC) with replicate as a random effect and choline (1.3 or 1.8 mM), follistatin and the interaction as fixed effects. Prior to statistical analysis of transcript abundance, transcript data were log transformed to normalize the data and then back transformed for presentation. Transcript abundance and blastocyst cell numbers were analysed using the GLM procedure in SAS (SAS Inst. Inc., Cary, NC) with replicate as a random effect and choline (1.3 or 1.8 mM), follistatin and the interaction as fixed effects. Developmental timing of blastocysts was analysed with a repeated measures model using the GLM procedure in SAS (SAS Inst. Inc., Cary, NC) with choline (1.3 or 1.8 mM), follistatin and the interaction as a fixed effects and time as a repeated measure. A P-value ≤ 0.05 was considered statistically significant and a P-value between 0.05 and 0.1 was considered a tendency. All data are presented as LS means ± SEM.

Results

In vitro *maturation choline and follistatin supplementation on oocyte competence endpoints*

Cumulus oocyte complexes matured in the presence of choline and/or follistatin did not differ in the percentage of cumulus cell expansion based on visual inspection ($P > 0.1$, Table 1). Transcript abundance of *CHPT1*, *CX43*, *EGFR1*, *FST* and *STAR* was not different between choline, follistatin or the combination of both in cumulus cells ($P > 0.1$, Table 2). When transcript abundance in oocytes was examined, *CEPT1* tended to be reduced in choline 1.8 mM x follistatin compared to choline 1.8 mM ($P = 0.07$, Table 3). Choline or follistatin supplementation did not impact oocyte *CEPT1* transcript abundance ($P > 0.1$). Oocytes matured with choline 1.3 mM alone had a tendency ($P = 0.1$) for *CPEB4* to be increased compared to the control group. A tendency was also observed for *CPEB4* to be reduced in oocytes matured with the combination of choline 1.3 mM and follistatin ($P = 0.08$) compared to oocytes supplemented with choline 1.3 mM (Table 3). No differences in transcript abundance were observed for *BMP15, COX2, CX43, GDF9, MTHFR* and *NANOG* in choline, follistatin or the combination in matured oocytes ($P > 0.1$, Table 3).

In vitro *maturation choline and follistatin supplementation on early embryonic development*

No differences were observed for cleavage rate of PZ after fertilization ($P > 0.9$; Figure 1A), cleaved embryos developing to blastocyst stage or PZ developing to blastocyst stage ($P > 0.9$; Figure 1B and 1C). In the subset of PZ that underwent time lapse

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Table 1. Cumulus cell expansion rate from COC supplemented with choline, follistatin, or a combination of choline and follistatin

Cumulus Cell Expansion	Treatment, %						*P*-Value		
	Control	Choline 1.3 mM	Choline 1.8 mM	Follistatin	Choline 1.3 mM x Follistatin	Choline 1.8 mM x Follistatin	Choline	Follistatin	Choline x Follistatin
Excellent/Good	91.0 ± 16.5	86.7 ± 19.6	86.0 ± 20.0	88.7 ± 18.3	88.0 ± 18.3	88.3 ± 15.3	0.98	0.98	0.99
Fair	9.8 ± 17.2	15.4 ± 20.8	15 ± 20.6	12.7 ± 19.2	13.5 ± 19.7	13.5 ± 19.7	0.98	0.98	0.99
Poor	0.0 ± 0.0	0.0 ± 0.0	8.6 ± 16.2	8.3 ± 15.0	5.5 ± 13.0	0.0 ± 0.0	1.00	0.99	0.99

Data presented as LS means ± SEM.

Table 2. Transcript abundance in cumulus cells from oocytes matured with choline, follistatin, or a combination of choline and follistatin

Gene	Treatment						*P*-Value		
	Control	Choline 1.3 mM	Choline 1.8 mM	Follistatin	Choline 1.3 mM x Follistatin	Choline 1.8 mM x Follistatin	Choline	Follistatin	Choline x Follistatin
CHPT1	3.52 ± 1.07	2.74 ± 0.83	3.83 ± 1.16	3.98 ± 1.20	3.03 ± 0.92	3.14 ± 0.95	0.67	0.97	0.84
CX43	1.55 ± 0.32	1.73 ± 0.35	2.70 ± 0.55	1.92 ± 0.39	2.43 ± 0.50	2.39 ± 0.49	0.19	0.39	0.51
EGFR1	2.42 ± 0.47	2.10 ± 0.40	3.15 ± 0.61	2.90 ± 0.56	2.55 ± 0.49	2.12 ± 0.41	0.75	0.96	0.24
FST	2.90 ± 0.75	2.49 ± 0.64	3.84 ± 0.99	3.02 ± 0.78	2.74 ± 0.71	2.56 ± 0.66	0.76	0.67	0.57
STAR	2.74 ± 1.50	4.80 ± 1.51	6.45 ± 3.52	3.57 ± 1.95	10.98 ± 5.99	6.34 ± 3.50	0.37	0.22	0.38

Transcript abundance was expressed as a fold change relative to the lowest expressing sample following comparative CT calculation; Data presented as LS means ± SEM.

Table 3. Transcript abundance in oocytes matured with choline, follistatin or a combination of choline and follistatin

Gene	Treatment						*P*-Value		
	Control	Choline 1.3 mM	Choline 1.8 mM	Follistatin	Choline 1.3 mM x Follistatin	Choline 1.8 mM x Follistatin	Choline	Follistatin	Choline x Follistatin
BMP15	7.80 ± 2.44	13.94 ± 4.35	8.35 ± 2.61	13.31 ± 4.16	5.58 ± 2.68	4.93 ± 1.54	0.19	0.53	0.16
CEPT1	5.56 ± 1.68	10.85 ± 3.28	7.31 ± 2.21	10.95 ± 3.31	7.30 ± 2.20	3.69 ± 1.12	0.19	0.58	*0.07*
CPEB4	6.61 ± 1.93	13.86 ± 2.78	7.66 ± 1.54	14.28 ± 2.86	9.55 ± 1.91	4.98 ± 1.00	0.10	0.96	*0.08*
COX2	3.80 ± 1.07	6.92 ± 1.95	5.37 ± 1.51	7.00 ± 1.97	5.55 ± 1.56	3.27 ± 0.92	0.38	0.88	0.14
CX43	3.68 ± 0.80	4.98 ± 1.09	6.74 ± 1.47	5.69 ± 1.25	6.90 ± 1.50	6.33 ± 1.38	0.26	0.2	0.49
GDF9	7.31 ± 2.33	14.10 ± 4.50	7.73 ± 2.47	13.70 ± 4.37	8.22 ± 2.62	4.62 ± 1.47	0.14	0.58	0.12
MTHFR	9.64 ± 2.80	12.40 ± 3.60	7.99 ± 2.32	12.77 ± 3.71	8.83 ± 2.57	5.05 ± 1.47	0.12	0.47	0.39
NANOG	6.20 ± 1.89	12.86 ± 3.93	7.03 ± 2.15	11.94 ± 3.65	8.95 ± 2.73	4.76 ± 1.45	0.13	0.9	0.16

Transcript abundance was expressed as a fold change relative to the lowest expressing sample following comparative CT calculation; Data presented as LS means ± SEM; significance $P < 0.05$; tendencies *P*-value 0.05–0.1.

incubation for developmental timing, duration of first cell cleavage to the two-cell stage was significantly longer ($P < 0.05$) from COCs cultured in choline 1.3 mM (Table 4), while no differences were observed with follistatin or the combination of choline and follistatin during IVM. Differences were not observed within either treatment or the interaction from the three-cell stage to the six-cell stage ($P > 0.1$; Table 4). Choline 1.8 mM supplementation delayed division to the seven-cell stage ($P < 0.05$), while follistatin supplementation tended to delay ($P = 0.06$) development to this cell stage (Table 4). Follistatin supplementation tended to delay development to the eight-cell stage ($P = 0.09$), while no other effects of choline supplementation or the interaction was observed (Table 4). Supplementation did not influence early embryonic development to the morulae, compacted morulae, early blastocyst or blastocyst stage ($P > 0.1$; Table 4). Blastocysts reached the expanding state later in the follistatin group ($P = 0.02$), while no influence was observed from choline supplementation or the interaction ($P > 0.1$; Table 4). It is important to note that development to the blastocyst stage in the choline 1.8 mM group and expanded blastocyst stage with choline 1.8 mM and the addition of follistatin did not occur within 190 hr of incubation.

Representative images of blastocysts stained with DAPI and CDX2 are shown in Figure 2A. Total blastocyst cell numbers were reduced compared to the control group when COC were

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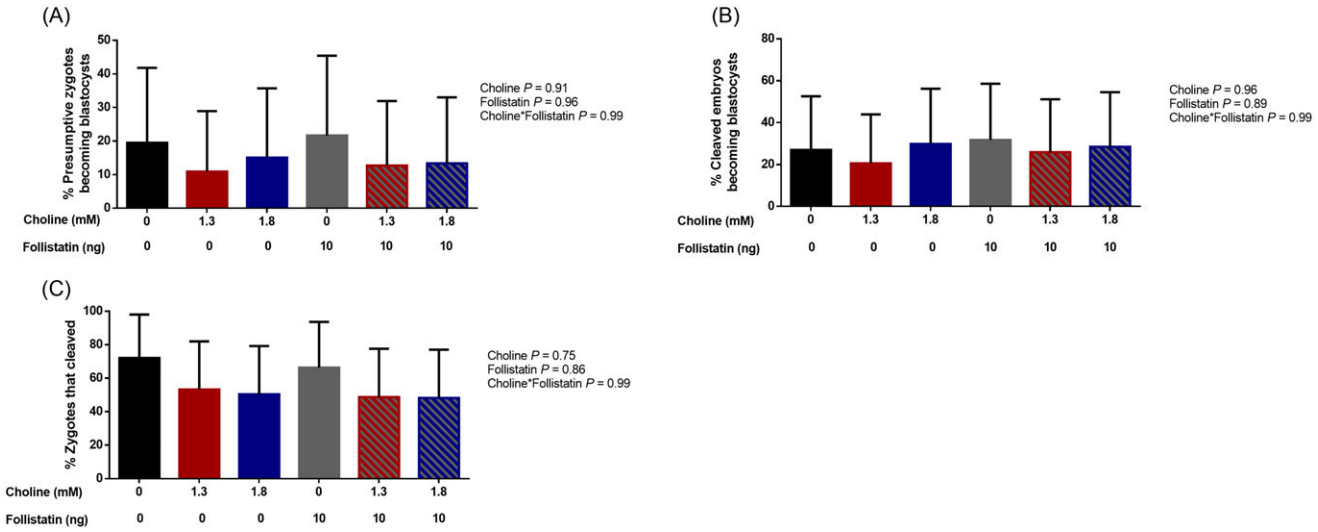

Figure 1. Effect of oocyte maturation medium supplemented with choline and/or follistatin on the percentage of PZ that cleaved after fertilization (A), percentage of cleaved embryos that became blastocysts (B) and percentage of zygotes that became blastocysts (C). Data are presented as LS mean ± SEM. The *P*-value for the main effect of choline, follistatin or the interaction are shown in each graph.

Table 4. Selected embryo timelapse data from COC supplemented with choline, follistatin or a combination of choline and follistatin

	Hour						***P*-Value**		
Cell Stage	Control	Choline 1.3 mM	Choline 1.8 mM	Follistatin	Choline 1.3 mM x Follistatin	Choline 1.8 mM x Follistatin	Choline	Follistatin	Choline x Follistatin
2-Cell	30.7 ± 1.7	35.4 ± 1.7	31.2 ± 1.6	30.9 ± 1.6	35.4 ± 1.6	31.9 ± 1.7	***0.01***	0.81	0.97
3-Cell	40.9 ± 3.1	41.4 ± 3.6	38.4 ± 3.2	41.0 ± 3.0	41.0 ± 3.1	43.2 ± 3.2	0.99	0.55	0.68
4-Cell	45.0 ± 2.9	43.0 ± 3.6	43.12 ± 3.4	43.3 ± 2.9	45.2 ± 3.0	45.9 ± 3.2	0.99	0.75	0.73
5-Cell	48.8 ± 4.1	46.4 ± 6.1	49.5 ± 4.8	52.3 ± 4.3	47.7 ± 4.8	56.2 ± 4.8	0.55	0.32	0.87
6-Cell	45.5 ± 4.4	46.0 ± 6.2	53.2 ± 4.7	53.8 ± 3.9	48.5 ± 4.7	60.6 ± 4.4	0.12	0.12	0.82
7-Cell	49.8 ± 5.5	48.5 ± 9.1	53.4 ± 7.8	55.0 ± 5.2	48.5 ± 5.2	79.4 ± 6.4	***0.02***	*0.06*	0.22
8-Cell	51.0 ± 5.5	51.2 ± 9.0	54.58 ± 9.0	56.7 ± 5.5	55.0 ± 7.0	76.5 ± 7.0	0.12	*0.09*	0.34
Morula	122.84 ± 4.9	111.8 ± 14.0	114.4 ± 14.0	109.7 ± 6.2	117.3 ± 7.0	126.1 ± 6.2	0.58	0.4	0.29
Comp. Morula	133.3 ± 5.5	124.7 ± 15.5	133.1 ± 15.5	125.8 ± 7.7	130.6 ± 7.7	147.5 ± 7.7	0.22	0.91	0.51
Early Blastocyst	148.0 ± 3.6	138.8 ± 10.3	154.3 ± 10.3	147.8 ± 5.9	145.8 ± 5.1	160.2 ± 5.9	0.13	0.63	0.83
Blastocyst	165.6 ± 4.0	146.1 ± 11.2	N/A	160.2 ± 6.5	160.8 ± 5.6	169.0 ± 7.9	0.44	0.99	0.19
Expanded Blastocyst	167.0 ± 3.3	166.9 ± 5.7	N/A	178.2 ± 3.3	180.0 ± 3.3	N/A	0.31	***0.02***	0.82

N/A- embryos failed to develop to these stages. Data presented as LS means ± SEM; significance $P < 0.05$; tendencies $P = 0.05$–0.1.

supplemented with the combination of follistatin with either choline 1.3 mM or 1.8 mM ($P < 0.01$, Figure 2B), while no differences were observed in either choline or follistatin supplemented groups alone. When the specific cell types were broken down into ICM and TE, there was a significant effect of choline supplementation with both cell types being reduced irregardless of concentration of choline compared to the control group ($P < 0.01$, Figure 2C and 2D). Cell numbers for both ICM and TE were not different ($P > 0.1$) in follistatin supplementation or the combination with choline.

Specific transcript abundance markers of epigenetic regulation and early embryonic development were analysed in pooled blastocysts from each treatment group. No differences were observed for *KDM2B* in choline, follistatin or the interaction ($P > 0.1$, Figure 3A). Choline 1.8 mM supplementation increased transcript abundance for *HDAC8*, *AMOT*, *NANOG*, *HDAC8, MBNL3, STAT1*, *STAT3, SAV1* and *SOX2* compared to the control group ($P < 0.05$, Figures 3C-J). Follistatin supplementation tended to reduce transcript abundance of *HNF4A*, *MBNL3* and *STAT3* (Figure 3B, F and H). An interaction was observed between choline

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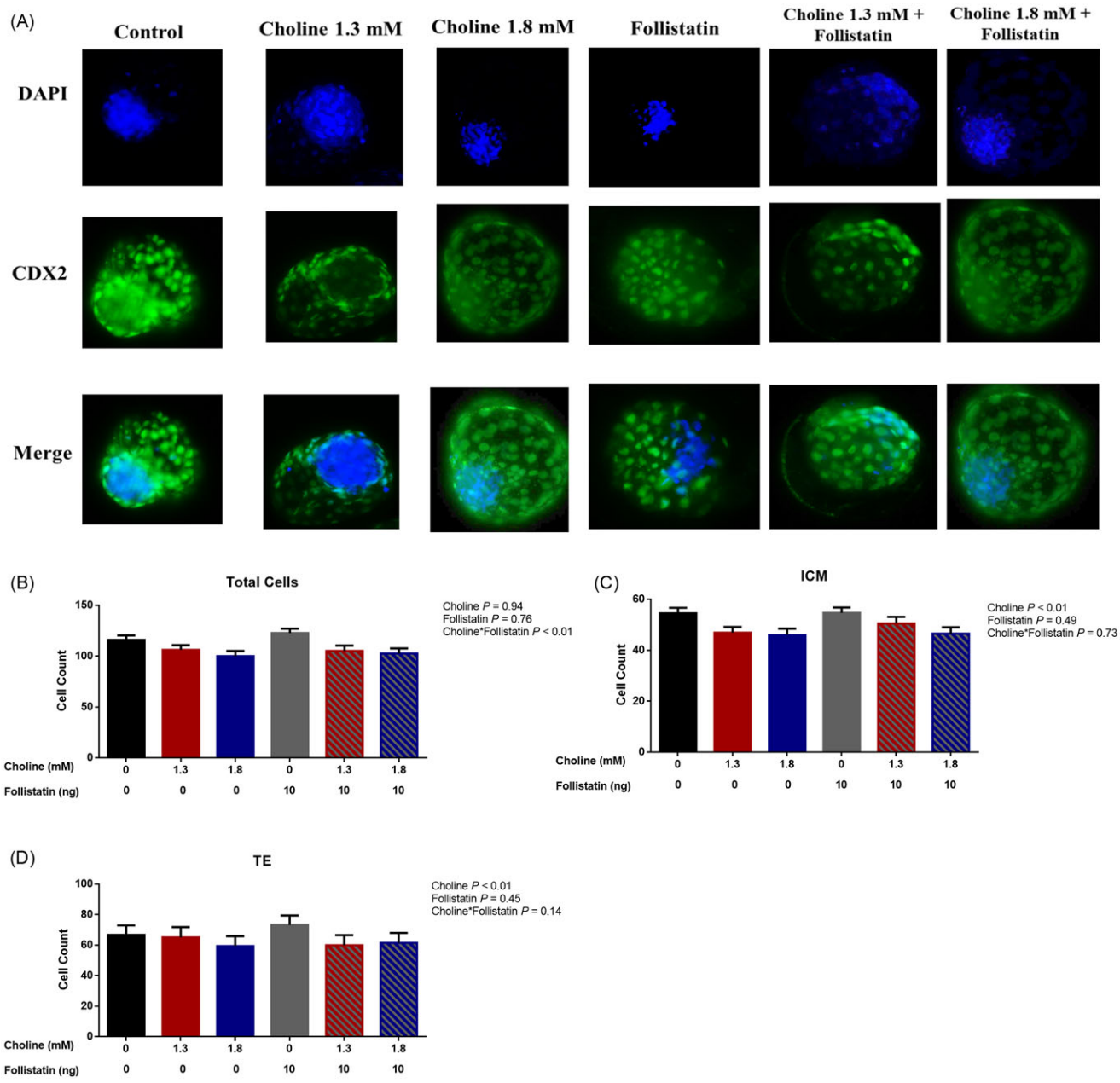

Figure 2. Representative images of blastocysts (A), total cell (B), inner cell mass (C) and trophectoderm (C) numbers of blastocysts collected at day 7.5 post-fertilization. Blastocysts were derived from oocytes supplemented with different treatments: control, choline 1.3 mM, choline 1.8 mM, follistatin, choline 1.3 mM x follistatin and choline 1.8 mM x follistatin, stained with DAPI (blue) or CDX2 (green) and a merge of the two staining methods. Data are presented as LS mean ± SEM. The *P*-value for the main effect of choline, follistatin or the interaction are shown in each graph.

and follistatin for increased transcript abundance in *HDAC8* and *SOX 2* in choline 1.8 mM x follistatin compared to choline 1.8 mM ($P < 0.05$, Figure 3E and 3J).

Discussion

The purpose of this experiment was to investigate the use of choline and follistatin supplementation during bovine oocyte maturation and the subsequent influence on early embryonic development *in vitro*. Research showed an impact of supplementing choline or follistatin during *in vitro* embryo culture, compared to COC cultured in basal maturation and fertilization medium (Estrada-Cortes *et al.*, 2020; Zhenhua *et al.*, 2017). Results from the current study demonstrate that supplementation with either choline or follistatin during *in vitro* oocyte maturation does not impact COC development. Supplementation of 1.8 mM choline with follistatin during oocyte maturation altered transcript abundance of markers as sociated to early embryonic development, while no significant differences were observed in cleavage or blastocyst developmental rate. The long-term influence of choline and follistatin during *in vitro* oocyte maturation increased expression of markers for early embryonic development in the blastocyst and may lead to improved embryonic development *in vivo* after embryo transfer.

Follistatin supplementation has been used in embryo culture medium, as it is part of the TGFβ superfamily that can either inhibit or increase specific ligands or growth factors necessary for

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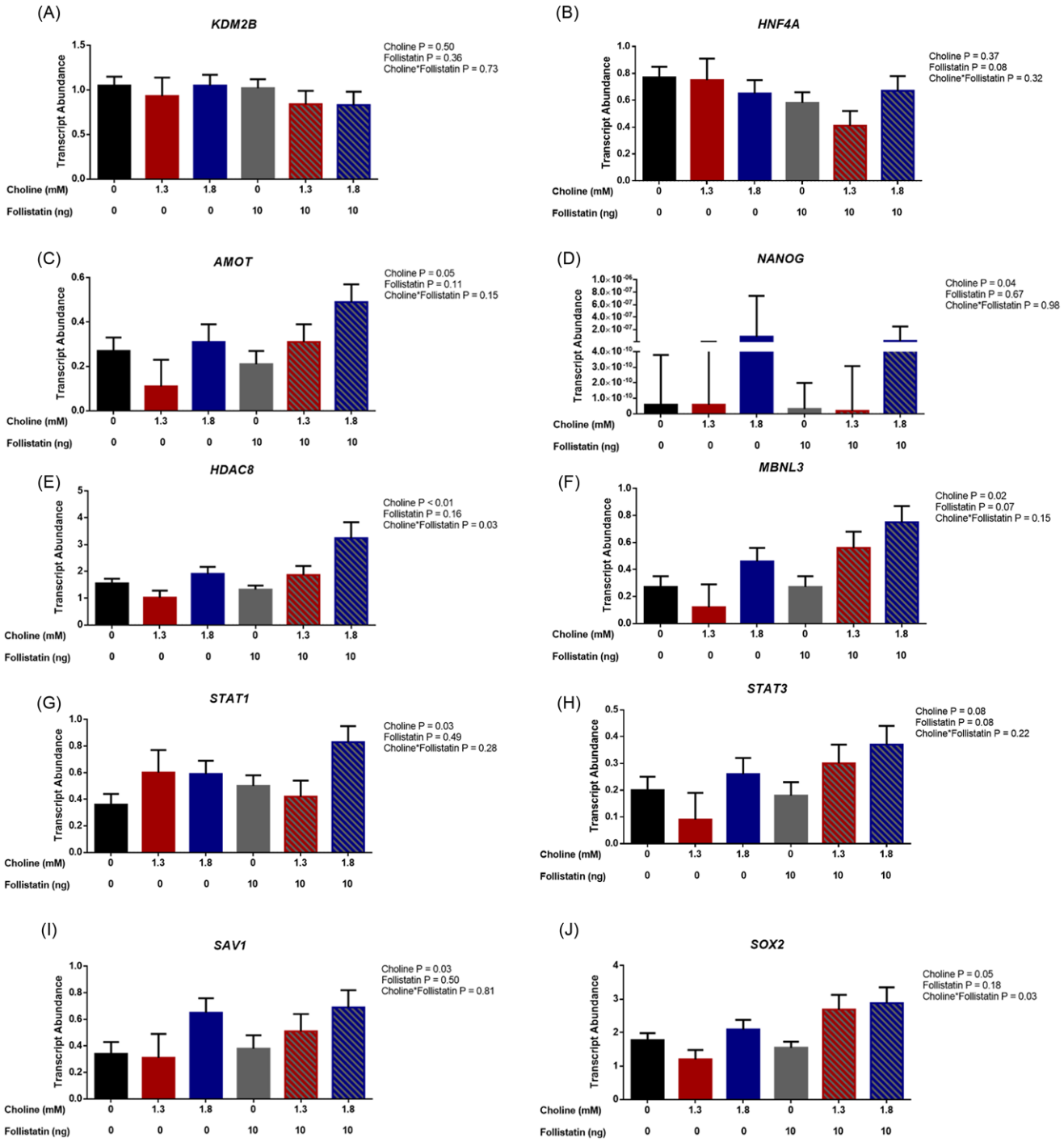

Figure 3. Effect of choline and follistatin supplementation in the oocyte maturation medium on transcript abundance of specific genes in blastocysts. Transcript abundance was expressed as a fold change relative to the lowest expressing sample following comparative CT calculation. Data are presented as LS mean ± SEM. The *P*-value for the main effect of choline, follistatin or the interaction is shown in each graph. Significant differences determined with $P < 0.05$ and tendencies reported with *P*-value between 0.05 and 0.1.

blastocyst development (Zhenhua *et al.*, 2017). During oocyte maturation, follistatin provides a beneficial impact to regulate activin signaling to improve oocyte maturity and capacity for fertilization (Izadyar *et al.*, 1998). Research investigating follistatin supplementation during IVP demonstrated a beneficial response on development of cleaved embryos to the blastocyst stage (Ashry *et al.*, 2018; Ashry *et al.*, 2020; Zhenhua *et al.*, 2017). In the current study, supplementation of follistatin during oocyte maturation did not increase transcripts associated with oocyte quality. There was an increase in transcript abundance of *STAT3* and *MBNL3* in the blastocyst, which are associated with early embryonic development. Increased abundance of *STAT3* can lead to improved pluripotency of the ICM (Meng *et al.*, 2015) while *MBNL3* is located within the hypoblast of the blastocyst and responsible for muscle differentiation in early fetal development (Lee *et al.*, 2007; Negron-Perez *et al.*, 2017).In this study, no differences were

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observed in total cell number or number of ICM or TE cells in blastocysts from COC treated with follistatin. Tendencies were observed for selected PZ cultured in a timelapse incubator to have delayed divisions of the 7th and 8th blastomere stage. Timing of blastocysts to reach the expanded state was also delayed when COC were treated with follistatin. Taken together, addition of follistatin during IVM influences embryonic development.

Choline is an important metabolite since it facilitates phospholipid synthesis for acetylcholine production and further regulation of DNA methylation (Jayaprakash *et al*., 2016). This metabolite was used extensively in dairy cattle through nutritional supplementation to increase production of phosphatidylcholine (Jayaprakash *et al*., 2016). Phosphatidylcholine is important in many physiological roles, including reproduction. Supplementation of rumen-protected choline to the dam during gestation reduced oxidative stress and altered metabolism in newborn calves (Swartz *et al*., 2022). Studies performed by Estrada-Cortes *et al*., 2020 and 2021, supplemented choline during IVP and demonstrated an impact on blastocyst quality and global DNA methylation that can lead to altered calf performance (Estrada-Cortes *et al*., 2020; Estrada-Cortes *et al*., 2021; Estrada-Cortes *et al*., 2019). Since choline appears to have an impact on many different aspects of development, addition of choline to IVM media could improve *in vitro* oocyte quality. In the current study, choline supplementation with 1.3 mM tended to increase *CPEB4* transcript abundance, which is an important regulator of cell cycle progression for the oocyte to move from the MI to MII phase during maturation (Ganz *et al*., 2017). While no other differences were observed in oocyte quality, it was important to determine if choline supplementation at this stage had long-term implications on early embryonic development.

Choline supplementation during embryo culture altered blastocyst DNA methylation, markers associated with early embryonic development and subsequent calf performance when the 1.3 mM and 1.8 mM concentration of choline was used(Estrada-Cortes *et al*., 2020; Estrada-Cortes *et al*., 2021). In this current study, when choline was supplemented during IVM no differences was observed in cleavage or blastocyst rate, supporting what has been previously reported with supplementation during IVP. Differences were observed in blastocyst transcript abundance that were derived from COC supplemented with 1.8 mM choline, specifically *AMOT*, *HDAC8*, *MBNL3*, *NANOG*, *SAV1*, *SOX2*, *STAT1* and *STAT3*. These transcripts have important roles in blastocyst epigenetic regulation and cellular differentiation. One specific transcript, *HDAC8,* is important for encoding a histone deacetylase enzyme, which is a major component for epigenetic regulation (Hosseini *et al*., 2015). Early muscle differentiation is modulated by *MBNL3* and is located within the hypoblast cells of the blastocyst (Lee *et al*., 2007; Negron-Perez *et al*., 2017). Transcripts *STAT1* and *STAT3* are important for regulating the cytokine response during early embryonic development (Estrada-Cortes *et al*., 2020). Pluripotency of the ICM is regulated by *NANOG*, *SOX2*, *STAT1* and *STAT3* (Negron-Perez *et al*., 2017; Wooldridge and Ealy, 2019). Development of TC is regulated by *AMOT* and *SAV1*, that also code important proteins involved in the Hippo signaling pathway (Negron-Perez and Hansen, 2018). Cell numbers of ICM and TE were reduced in blastocysts supplemented with choline, which is contrary to what was observed in the transcript abundance. The increased transcript abundance of *NANOG*, *STAT1*, *STAT3* and *SOX2* could lead to later pluripotency of the ICM., TE and total cell numbers were reduced in the choline treatment at the blastocyst stage. Combination of choline and follistatin reduced total cell numbers, but no difference was observed in ICM or TE numbers. Interestingly, the transcript abundance of pluripotency markers of ICM and TE were increased, while there was a reduction in cell numbers in the choline and follistatin treatments combined. This was not expected, and further research is warranted to understand the differences observed.

Combination of choline and follistatin during IVM tended to reduce transcript abundance of *CPEB4* and *CEPT1* in the oocyte. An important regulator of lysophosphatidylcholine conversion to phosphatidylcholine is *CEPT1* (Ganz *et al*., 2017). This was an interesting result, as supplementation of choline alone tended to increase *CPEB4* in the oocytes. Supplementing both metabolites together could be acting in an anti-synergistic way to reduce the abundance of these transcripts. No other differences were observed in the transcript abundance of oocytes. Supplementation of choline and follistatin together during IVM had a long-term impact on the blastocyst when analyzing transcript markers associated with quality. Choline 1.8 mM and follistatin supplementation during oocyte maturation increased transcript abundance of *HDAC8* and *SOX2* in the blastocyst. This increased abundance of transcripts could be due to epigenetic regulation occurring within the matured oocyte that leads to changes in the blastocyst. Timing to the 7- and 8- cell stage was delayed by the combination of 1.8 mM choline and follistatin compared to the rest, but no differences in timing to later stages of development were observed. Follistatin supplementation during oocyte maturation had a positive effect on the total cell numbers in blastocysts, however, when choline was added into the medium a reduction in total cell numbers was observed. This blastocyst cell number reduction could be due to molecular changes during oocyte maturation, but further research is warranted to understand these effects. Similar cleavage and blastocyst rates were also observed with the combination of these two different metabolites.

In conclusion, this study was novel in the investigation of supplementing choline and follistatin together during bovine IVM with subsequent impacts on blastocyst development using basal IVF and IVC media. Supplementation did not have a direct impact on the immediate quality of the oocyte; however, supplementation with choline 1.8 mM or the combination of choline 1.8 mM and follistatin together increased transcript abundance of blastocyst quality markers. Differences in blastocyst transcript abundance observed could be related to potential changes in DNA methylation, causing altered transcript abundance in later development. Continued research is needed to fully understand the long-term influence of supplementing these different metabolites on the methylation status of the oocyte and long-term influences in a conceptus.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199424000145

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Competing interests. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical standard. Material used for this project was abattoir-derived only and no review was required by an Animal Welfare and Ethical Review Body.

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