

# Effect of arachidonic acid on pre- and post-hatching *in vitro* bovine embryo development

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## ABSTRACT

**Context.** Arachidonic acid (AA) is the precursor of prostaglandins, which may play autocrine roles during early embryo development. **Aims.** To test the developmental effects of addition of AA to pre- and post-hatching culture media on *in vitro*-produced bovine embryos. **Methods.** Pre-hatching effects of AA were tested by culturing bovine zygotes in synthetic oviductal fluid (SOF) supplemented with 100 or 333  $\mu\text{M}$  AA. Post-hatching effects of AA were tested by culturing Day 7 blastocysts in N2B27 supplemented with 5, 10, 20 or 100  $\mu\text{M}$  AA up to Day 12. **Key results.** Pre-hatching development to blastocyst was completely abrogated at 333  $\mu\text{M}$  AA, whereas blastocyst rates and cell numbers were not altered at 100  $\mu\text{M}$  AA. Impaired post-hatching development was observed at 100  $\mu\text{M}$  AA, whereas no effect on survival rates was noted at 5, 10 and 20  $\mu\text{M}$  AA. However, a significant reduction in Day 12 embryo size was observed at 10 and 20  $\mu\text{M}$  AA. Hypoblast migration, epiblast survival and formation of embryonic-disc-like structures were unaffected at 5–10  $\mu\text{M}$  AA. AA exposure downregulated the genes *PTGIS*, *PPARG*, *LDHA* and *SCD* in Day 12 embryos. **Conclusions.** Pre-hatching embryos are mostly irresponsive to AA, whereas AA was observed to have negative effects during early post-hatching development. **Implications.** AA does not improve *in vitro* bovine embryo development and is not required up to early post-hatching stages.

**Keywords:** arachidonic, bovine, conceptus, elongation, embryo development, lipids, *PPARG*, prostaglandin, *SCD*, trophectoderm.

## Introduction

Preimplantation embryo development relies on the nutrients, hormones and growth factors present in oviductal and uterine fluids. The media developed for *in vitro* embryo culture have been designed to roughly mimic the conditions an embryo encounters through its journey from the oviduct to the uterus (Holm *et al.* 1999). Diverse embryo culture media are able to sustain embryo development from the zygote stage to blastocyst formation, although embryo quality, defined as the ability to establish a viable pregnancy, is reduced compared to *in vivo* developed blastocysts (van de Leemput *et al.* 1999; Dieleman *et al.* 2002; Rizo *et al.* 2002). Around blastocyst hatching, embryonic requirements change and conventional embryo culture media are unable to support embryo development (reviewed by Perez-Gomez *et al.* 2021). Recently, N2B27 medium has been found to be able to sustain early post-hatching development, attaining critical developmental landmarks such as complete hypoblast migration and the formation of embryonic-disc-like (ED-like) structures (Ramos-Ibeas *et al.* 2020). Compared to conventional embryo culture media, such as synthetic oviductal fluid (SOF), N2B27 – originally designed for stem cell culture (Ying and Smith 2003) – exhibits a more complex composition, including hormones and diverse compounds such as lipids.

Lipids constitute a major component of the uterine fluid where embryos develop (Ribeiro *et al.* 2016a; Simintiras *et al.* 2019), playing essential structural roles in biological membranes, being involved in metabolic pathways and acting as precursors of lipid signalling molecules. Prostaglandins (PGs) constitute a group of lipid signalling molecules known to play critical roles during gestation by promoting luteolysis (PGF2 $\alpha$ ) or corpus

luteum maintenance (PGE<sub>2</sub>) (Reynolds *et al.* 1983; Lee *et al.* 2012). Besides these well-known roles, PGs have been suggested to play relevant functions before maternal recognition of pregnancy, during early conceptus elongation (Dorniak *et al.* 2011), a period of embryo development exclusive to ungulates that spans blastocyst hatching to implantation. Several observations suggest that conceptus-derived prostaglandins may play paracrine (i.e. signalling to the endometrium) and autocrine (i.e. signalling in the conceptus) roles during conceptus elongation. As conceptus elongation progresses, the concentrations of PGs (PGF<sub>2</sub> $\alpha$  and PGE) and other prostanoids such as prostacyclin (PGI<sub>2</sub>), mainly produced by the conceptus, increase in the uterine fluid (Ulbrich *et al.* 2009). In turn, PGE<sub>2</sub> receptors (PTGER2 and PTGER4) and the PGF<sub>2</sub> $\alpha$  receptor PTGFR are abundant in the conceptus trophoctoderm and endometrial epithelia (Dorniak *et al.* 2011), and the PGI<sub>2</sub> receptor PTGIR and the nuclear PG receptors PPARs are also present in ovine and bovine endometrial epithelia and conceptuses (Cammass *et al.* 2006; Ribeiro *et al.* 2016b).

PGs and all other prostanoids derive from arachidonic acid (AA) by the cyclooxygenase pathway. AA is a polyunsaturated omega-6 fatty acid 20:4( $\omega$ -6) that can be acquired from the diet or synthesised from the essential  $\alpha$ -linolenic acid through an initial step of desaturation by the  $\Delta^6$ -desaturase FADS2, followed by 2-carbon elongation by the elongase ELOV5, then desaturation by the  $\Delta^5$ -desaturase FADS1 (Sergeant *et al.* 2016). However, it is unclear if the blastomeres of preimplantation embryos are able to convert  $\alpha$ -linolenic into AA, and AA is not present in serum-free media such as plain SOF – which does not contain any lipid compounds – or N2B27. AA concentrations in the uterine fluid where embryos develop are responsive to progesterone (Simintiras *et al.* 2019). AA can be metabolised to PGs by elongated bovine conceptuses (Lewis *et al.* 1982; Hwang *et al.* 1988), and AA content in the embryo increases significantly from pre-hatching (Day 7) to elongating (Day 14) stages (Menezo *et al.* 1982), suggesting that AA may be required for embryo development.

The developmental effects of the addition of AA to conventional (pre-hatching) embryo culture have been scarcely explored. Two early studies observed that the addition of AA at 50 ng/mL (~164 nM) to BECM medium increased morula formation rate without achieving development to blastocyst in a single embryo culture system (Lim and Hansel 1996) and increased morula formation rate without significantly improving blastocyst rate when combined with glutathione in a group culture system (Lim and Hansel 2000). Given the novelty of the post-hatching culture system (Ramos-Ibeas *et al.* 2020), the addition of AA to post-hatching culture has not been tested. Testing the addition of AA following hatching is particularly relevant, as this developmental period is particularly susceptible to embryonic loss in cattle (Dunne *et al.* 2000; Diskin and Morris 2008; Berg *et al.* 2010), prostaglandin secretion by the conceptus increases significantly

along conceptus elongation (Lewis *et al.* 1982; Ulbrich *et al.* 2009), and the expression of different prostaglandin receptors which may mediate autocrine signalling also increases in the elongating conceptus (Cammass *et al.* 2006; Dorniak *et al.* 2011; Ribeiro *et al.* 2016b). In this study, we have analysed the developmental effects of the addition of AA to pre- and post-hatching culture media on *in vitro*-produced bovine embryos in two independent experiments.

## Materials and methods

### *In vitro* production of bovine blastocysts

Bovine embryos were produced *in vitro* as previously described (Bermejo-Alvarez *et al.* 2011) with specific modifications in the culture system tailored to reliably test the addition of a lipophilic compound naturally present in serum. Briefly, immature cumulus-oocyte complexes (COCs) were obtained by aspirating 2–8 mm follicles from bovine ovaries collected at local slaughterhouses. COCs with homogeneous cytoplasm and intact cumulus cells were selected and matured for 24 h in groups of 50 in 500  $\mu$ L of TCM-199 medium supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng/mL epidermal growth factor (EGF) at 38.5°C under an atmosphere of 5% CO<sub>2</sub> in air with maximum humidity. For *in vitro* fertilisation (IVF), matured COCs were inseminated with frozen-thawed bull sperm separated through a gradient of 40–80% BoviPure (Nidacon), at a final concentration of 10<sup>6</sup> spermatozoa/mL. Gametes were co-incubated in 500  $\mu$ L of Tyrode's albumin lactate pyruvate (TALP) medium supplemented with 10 mg/mL heparin at 38.5°C in a water saturated atmosphere of 5% CO<sub>2</sub>. Semen from the same bull was used for all experiments to avoid a possible confounding bull effect on developmental rates and to reduce inter-replicate variation. At approximately 20 h post-insemination (hpi), presumptive zygotes were denuded by vortexing for 3 min and cultured *in vitro*. To contrast the hypothesis of a potential beneficial effect of AA addition to the media used for *in vitro* culture of bovine embryos, two experiments were conducted.

For Experiment 1 (effect of AA during pre-hatching culture), presumptive zygotes were cultured in groups of ~50 in 500  $\mu$ L of SOF (Holm *et al.* 1999) supplemented with 3 g/L of bovine serum albumin (BSA) essentially fatty acid free (Sigma A6003; Sigma-Aldrich) and different concentrations of AA as described below. The use of mineral oil during culture was avoided to impede the migration of AA from the aqueous culture medium to the mineral oil, and serum was not used to avoid the addition of the AA naturally present in blood. To prevent media evaporation, 4-well dishes were used and the inner cavity surrounding the wells was filled with distilled water. AA (Sigma A3611; Sigma-Aldrich) addition was tested at two concentrations (100 or 333  $\mu$ M) resulting in three experimental groups: no AA (Control), AA

100  $\mu\text{M}$  and AA 333  $\mu\text{M}$ . As AA was dissolved in ethanol, an equivalent amount of ethanol (0.3%) was added to the control group. Half of the culture medium was replaced by fresh medium on Days 3 and 6 post-fertilisation, and culture took place at 38.5°C in an atmosphere of 5%  $\text{CO}_2$ , 5%  $\text{O}_2$  and 90%  $\text{N}_2$  with maximum humidity. Developmental rates were recorded at 48 hpi (cleavage rate) and at Day 7 and Day 8. At Day 8, blastocysts were fixed in a solution of 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature (RT) and stored in PBS-1% BSA at 4°C until analysis.

For Experiment 2 (effect of AA during post-hatching culture), presumptive zygotes were cultured in 25  $\mu\text{L}$  drops of SOF medium supplemented with 5% fetal calf serum under mineral oil up to Day 7. Beyond Day 7, embryo culture was performed as described in Ramos-Ibeas *et al.* (2020) but starting exposure to N2B27 medium at an earlier stage (Day 7), a modification that improves post-hatching developmental rates (Ramos-Ibeas *et al.* 2023). N2B27 medium is composed of 1:1 Neurobasal and DMEM/F12 media supplemented with penicillin/streptomycin, 2 mM glutamine, N2 and B27 supplements (Gibco). A detailed comparison between SOF and N2B27 media composition can be found in Ramos-Ibeas *et al.* (2020). Day 7 blastocysts (6–15/well) were transferred to 500  $\mu\text{L}$  N2B27, and AA addition was tested at four concentrations (5, 10, 20 and 100  $\mu\text{M}$ ), resulting in five experimental groups: no AA (Control), AA 5  $\mu\text{M}$ , AA 10  $\mu\text{M}$ , AA 20  $\mu\text{M}$  and AA 100  $\mu\text{M}$ . As AA was dissolved in ethanol, an equivalent amount of ethanol (0.3%) was added to the control group. Half of the media was replaced at Day 9 and Day 11, and culture took place at 38.5°C in an atmosphere of 5%  $\text{CO}_2$ , 5%  $\text{O}_2$  and 90%  $\text{N}_2$  with maximum humidity. At Day 12, survival rates were recorded (alive embryos were able to maintain the blastocoel, whereas dead embryos collapsed, see Supplementary Fig. S1), pictures were taken on a stereomicroscope (Zeiss Stemi 305) to measure embryo diameter, and surviving embryos were snap frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until transcriptional analysis or fixed in a solution of 4% PFA in PBS for 15 min at RT and stored in PBS-1% BSA at 4°C until immunofluorescence analysis.

### Immunofluorescence analysis

Fixed embryos were washed in PBS-1% BSA, permeabilised in 1% Triton X-100 in PBS for 15 min at RT and blocked in 10% Donkey Serum-0.02% Tween 10 in PBS for 1 h at RT. Then embryos were incubated overnight at 4°C with primary antibodies to detect trophoctoderm (CDX2, Biogenex MU392A-UC, 1:100 dilution), hypoblast (SOX17, R&D AF1924, 1:100 dilution) or inner cell mass/epiblast (SOX2, Invitrogen 14-9811-80, 1:100 dilution) cells. Day 8 blastocysts were immunostained to detect trophoctoderm and inner cell mass, and trophoctoderm, hypoblast and epiblast lineages were detected on Day 12 embryos. After four washes in PBS-1% BSA, embryos were incubated in the appropriate

secondary Alexa-conjugated antibodies (Donkey anti-rat IgG Alexa Fluor 488, Donkey anti-goat IgG Alexa Fluor 555 and Donkey anti-mouse IgG Alexa Fluor 647, Life Technologies) and DAPI for 1 h at RT, followed by four washes in PBS-1% BSA. Finally, embryos were mounted on incubation chambers as described in Bermejo-Alvarez *et al.* (2012) to preserve their 3D structure and imaged using structured illumination equipment composed of a Zeiss Axio Observer microscope coupled to ApoTome.2 (Zeiss). Hypoblast migration was considered complete when all the inner surface of the trophoctoderm was covered by SOX17+ cells. Epiblast survival was identified by the presence of SOX2+ cells in the embryo, whereas ED-like formation was identified by the presence of a compact structure containing SOX2+ cells.

### Transcriptional analysis by quantitative PCR

Poly(A) mRNA was extracted from five pools of three Day 12 embryos of each group (Control, AA 5  $\mu\text{M}$  and AA 10  $\mu\text{M}$ ) using the Dynabeads mRNA Purification kit (Life Technologies) following the manufacturer's instructions with minor modifications (Bermejo-Alvarez *et al.* 2011). Briefly, 100  $\mu\text{L}$  of lysis buffer was added to the sample and incubated at RT for 10 min with gently shaking. Then, 20  $\mu\text{L}$  of beads was added and samples were incubated at RT for 5 min with gently shaking, allowing the formation of bead/mRNA complexes. Finally, bead/mRNA complexes were washed twice in washing buffer A and twice in washing buffer B and resuspended in 10 mM Tris-HCl pH 7.5. Immediately after extraction, samples were treated with DNase (Promega) at 37°C for 5 min followed by enzyme denaturation at 90°C for 5 min. Retrotranscription reaction was carried out with qScript cDNA Supermix (Quantabiosciences) in a total volume of 20  $\mu\text{L}$ . Tubes were incubated at 25°C for 5 min followed by 42°C for 60 min and 85°C for 5 min.

Quantitative PCR (qPCR) analysis was conducted in duplicate for all genes of interest. Prior to the analysis, all primers used (Table 1) were tested to display qPCR efficiencies above 0.9. qPCR was performed by adding 2  $\mu\text{L}$  of diluted cDNA of each sample to the PCR mix (GoTaq qPCR Master Mix, Promega) containing the specific primers. The comparative cycle threshold (CT) method was used to quantify expression levels. Fluorescence was acquired in each cycle to determine the threshold cycle. According to the comparative CT method, the  $\Delta\text{CT}$  value was determined by subtracting the endogenous control H2AFZ CT value – tested for stability in previous publications (Bermejo-Alvarez *et al.* 2010a; Bermejo-Alvarez *et al.* 2011) – of each sample from the CT value of each gene in the sample.  $\Delta\Delta\text{CT}$  was calculated using the highest CT group value (i.e. the group with the lowest target expression) as an arbitrary constant to be subtracted from all other sample values. Fold changes in the relative gene expression of the target were determined using the formula  $2^{-\Delta\Delta\text{CT}}$ .

**Table 1.** Details of primers used for qPCR.

Gene	Primer sequence (5'–3')	Fragment size (bp)	GenBank accession no.
H2AFZ	AGGACGACTAGCCATGGACGTGTG CCACCACCAGCAATTGTAGCCTTG	209	NM_174809.2
IFNT2	GCTATCTCTGTGCTCCATGAGATG AGTGAGTTCAGATCTCCACCCATC	359	NM_001015511.3
PTGS2	CTGGCGGTAGGAATCTTCCA CCGAAAGTGCTAGGCTTCCA	349	NM_174445.2
PTGES	CGCGCTGCTGGTCATCAA CTGAGGCAGCGTTCACAT	190	NM_174443.2
PTGIS	GAGCAGAGCAGCCATATCA GGGACTCAGGAAGGGAAAGAG	185	NM_174444.1
PTGFR	GGTCATCCAGCTTCTGGGTAT AACAGCGTCTGGTACACACATA	227	NM_181025.3
PPARG	GCCCAAGTTCGAGTTTGCTG AGGCTTG CAGCAGATTGTCT	155	NM_037343.1
SCD	CCCACTCGTGCCATGGTAT TGGCAGCCATGCAATCAATG	300	NM_174809.2
CS	ATCCTCCTAGAGCAGGGCAA TGTGCTCATGGACTTGGGTC	204	NM_001044721.1
LDHA	TTCTTAAGGAAGAACATGTC TTCACGTTACGCTGGACCAA	310	NM_174099.2

## Statistical analysis

Data were analysed using the statistical software SigmaStat (Jandel Scientific). Cleavage, blastocysts and Day 8 to Day 12 survival rates and cell numbers were analysed by one-way ANOVA, whereas differences in complete hypoblast migration, epiblast survival and ED-like formation were analysed by Chi-square test.

## Results

AA addition during conventional *in vitro* culture (i.e. from Day 1 zygotes to Day 8 blastocysts) exerted a dose-dependent effect on embryo development (Table 2). Cleavage rates at 48 h after insemination – embryos cleaved out of presumptive zygotes in culture,  $n = 263$  – were not affected by AA addition up to 333  $\mu\text{M}$ , but blastocyst formation was completely abolished when embryos were exposed to AA at 333  $\mu\text{M}$ . In contrast, the addition of AA at 100  $\mu\text{M}$  did not impact negatively embryo development up to the blastocyst

stage, as developmental rates – embryos developing to blastocyst out of presumptive zygotes in culture – were comparable to the control (Table 2). Lineage allocation and cell proliferation were also unaffected by the addition of AA at 100  $\mu\text{M}$ , as no differences were noted in total, inner cell mass or trophectoderm cell numbers in Day 8 blastocysts cultured in SOF supplemented with 100  $\mu\text{M}$  AA or without AA (Table 3, Fig. 1a).

A dose-dependent effect of AA exposure during post-hatching *in vitro* culture (i.e. from Day 7 to Day 12) was evidenced in Day 12 survival rates (Table 4). Exposure to 100  $\mu\text{M}$  AA was not compatible with post-hatching embryo development, contrasting with the lack of a negative effect observed upon AA supplementation at that concentration from Day 1 to Day 8. AA concentrations below 20  $\mu\text{M}$  (5, 10 or 20  $\mu\text{M}$ ) did not reduce Day 7 survival rate to Day 12. However, Day 12 embryo diameter was gradually reduced in the presence of AA, being significantly diminished in 10 or 20  $\mu\text{M}$  groups compared to the control (Table 5). Despite the reduction in Day 12 embryo size at 10  $\mu\text{M}$ , lineage

**Table 2.** Developmental rates of zygotes cultured without AA (control) or in the presence of 100 or 333  $\mu\text{M}$  AA.

Group	Replicates	Zygotes	Cleaved	Day 7 blastocysts	Day 8 blastocysts
Control	4	156	85.6 $\pm$ 3.7% (125) <sup>a</sup>	25.3 $\pm$ 1.4% (37) <sup>a</sup>	32.2 $\pm$ 2.5% (47) <sup>a</sup>
AA 100 $\mu\text{M}$	4	154	83.1 $\pm$ 3.9% (128) <sup>a</sup>	22.7 $\pm$ 3.3% (35) <sup>a</sup>	28.6 $\pm$ 1.7% (44) <sup>a</sup>
AA 333 $\mu\text{M}$	3	73	84.9 $\pm$ 3.6% (62) <sup>a</sup>	0 $\pm$ 0 (0) <sup>b</sup>	0 $\pm$ 0 (0) <sup>b</sup>

Developmental rates (out of zygotes cultured) are represented as mean  $\pm$  s.e.m. (number). Different superscript letters within each column indicate statistically significant differences based on ANOVA ( $P < 0.05$ ).

**Table 3.** Number of total (DAPI+), inner cell mass (ICM, SOX2+) and trophectoderm (TE, CDX2+) cells in Day 8 blastocysts from control and AA 100  $\mu$ M groups, represented as mean  $\pm$  s.e.m. No significant differences were found.

Group	n	Total	ICM	TE
Control	13	80.2 $\pm$ 11.4	17.5 $\pm$ 2.7	62.7 $\pm$ 10.0
AA 100 $\mu$ M	14	87.5 $\pm$ 9.8	19.9 $\pm$ 2.2	67.6 $\pm$ 8.0

development in Day 12 embryos was comparable between 0, 5 and 10  $\mu$ M groups (Table 6, Fig. 1b). Complete hypoblast migration was observed in about half of the embryos in all groups, epiblast survival ranged from ~65–80% and the development of an ED-like structure was observed in roughly half of the embryos analysed irrespective of the concentration of AA (0–10  $\mu$ M).

To determine the possible molecular changes triggered by AA exposure, the expression level of genes related to pregnancy recognition (*IFNT2*), prostaglandin synthesis (*PTGS2*, *PTGES* and *PTGIS*), prostaglandin signalling (*PTGFR*), lipid signalling and metabolism (*PPARG* and *SCD*), and Krebs cycle and anaerobic glycolysis (*CS* and *LDHA*) were determined by qPCR on Day 12 embryos from control, AA 5  $\mu$ M and AA 10  $\mu$ M groups (Fig. 2). *PTGIS* and *PPARG* were significantly upregulated in Day 12 embryos cultured without AA

**Table 4.** Developmental rates in the post-hatching culture system of Day 7 blastocysts cultured without AA or in the presence of 5, 10, 20 or 100  $\mu$ M AA up to Day 12.

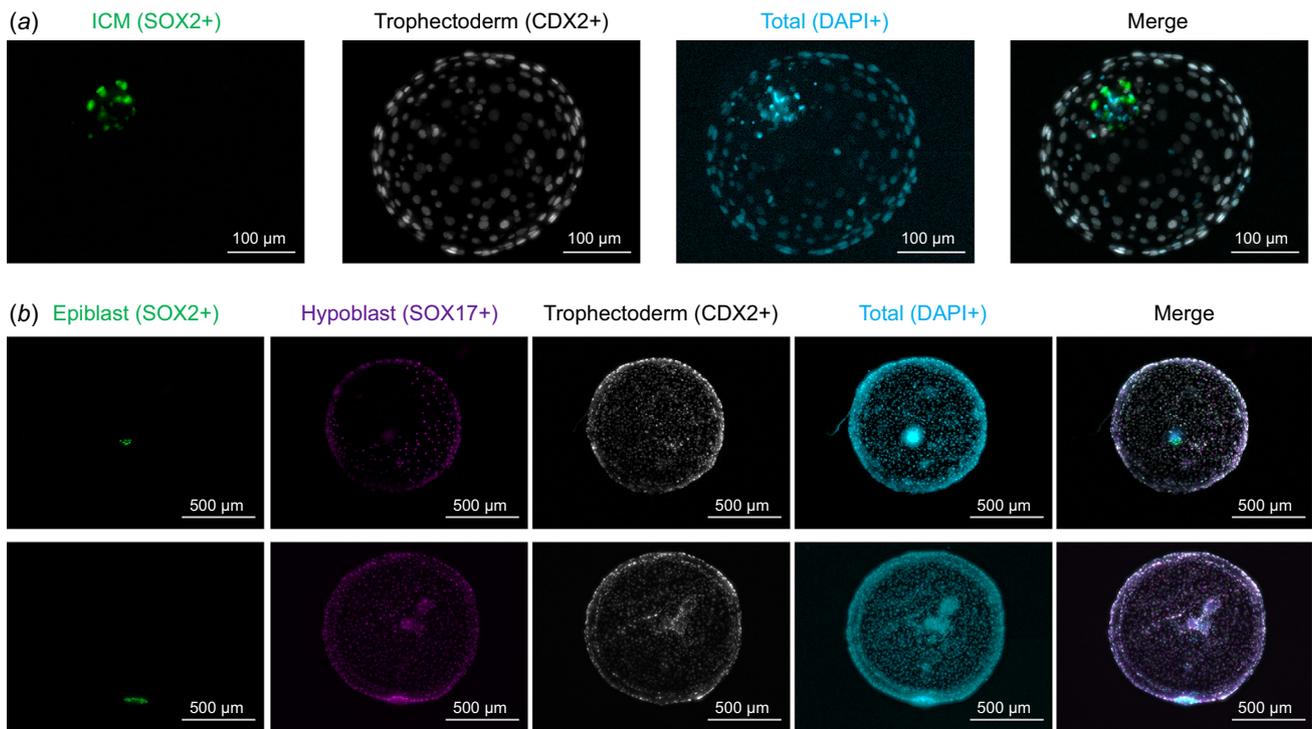
Group	Replicates	Day 7 blastocysts	Day 12 blastocysts
Control	11	106	85.8 $\pm$ 4.4% (91) <sup>a</sup>
AA 5 $\mu$ M	6	109	84.4 $\pm$ 4.8% (92) <sup>a</sup>
AA 10 $\mu$ M	6	74	89.2 $\pm$ 3.1% (66) <sup>a</sup>
AA 20 $\mu$ M	3	43	72.1 $\pm$ 4.2% (31) <sup>a</sup>
AA 100 $\mu$ M	2	19	0% (0) <sup>b</sup>

Developmental rates are represented as mean  $\pm$  s.e.m. (number). Different superscript letters indicate statistically significant differences based on ANOVA ( $P < 0.05$ ).

compared with those cultured at 5  $\mu$ M AA. *SCD* and *LDHA* were significantly upregulated in Day 12 blastocysts cultured without AA compared with those cultured at 10  $\mu$ M. No significant differences were noted for the other genes analysed.

## Discussion

The addition of lipid compounds to improve *in vitro* culture conditions has been scarcely explored. Conventional pre-hatching culture systems – based on microdrop culture – are



**Fig. 1.** Representative images of immunohistochemistry to detect embryonic lineages at different developmental days. (a) Day 8 blastocysts were stained for SOX2 (inner cell mass) and CDX2 (trophectoderm), nuclei were counterstained with DAPI. (b) Day 12 embryos were stained for SOX2 (epiblast), SOX17 (hypoblast, partial migration in upper image, complete migration in lower image) and CDX2 (trophectoderm), nuclei were counterstained with DAPI. Pictures are maximum projections of z-stacks.

**Table 5.** Effect of different AA concentrations on post-hatching embryo growth.

Group	N	Ø (mm)
Control	81	0.71 ± 0.05 <sup>a</sup>
AA 5 µM	64	0.64 ± 0.04 <sup>ab</sup>
AA 10 µM	66	0.51 ± 0.04 <sup>bc</sup>
AA 20 µM	20	0.32 ± 0.02 <sup>c</sup>

Embryo diameter is represented as mean ± s.e.m. Different superscript letters indicate statistically significant differences based on ANOVA ( $P < 0.05$ ).

**Table 6.** Effect of different AA concentrations on the development of specific lineages.

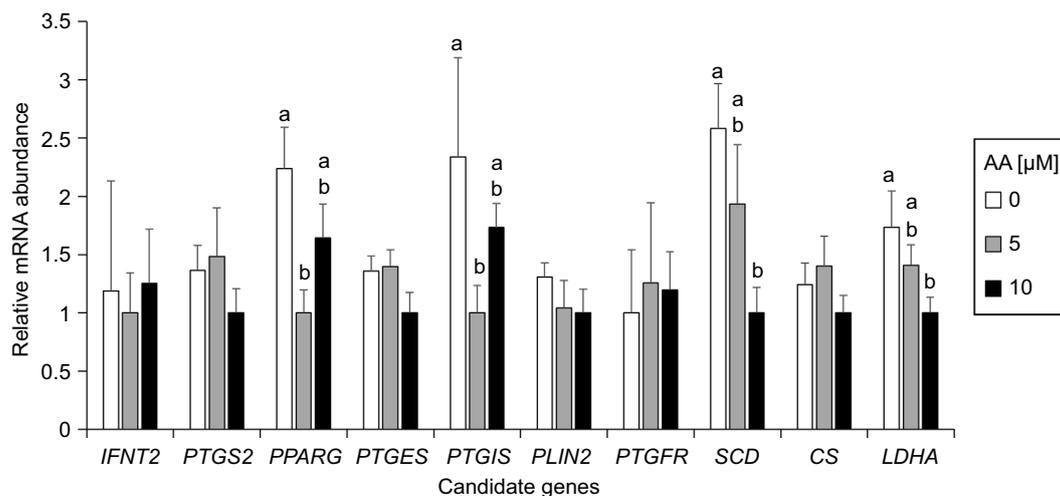
Group	Hypoblast migration (%)	Epiblast survival (%)	ED-like structure (%)
Control	18/40 (45.00) <sup>a</sup>	30/40 (75.00) <sup>a</sup>	22/40 (55.00) <sup>a</sup>
AA 5 µM	15/29 (51.72) <sup>a</sup>	23/29 (79.31) <sup>a</sup>	15/29 (51.72) <sup>a</sup>
AA 10 µM	22/35 (62.86) <sup>a</sup>	23/35 (65.71) <sup>a</sup>	17/35 (48.57) <sup>a</sup>

Different superscript letters indicate statistically significant differences within each column based z-test ( $P < 0.05$ ).

not suited for testing such compounds, as the overlying mineral oil captures the lipid compounds from the aqueous media. Being the precursor of PGs and all other prostanoids, AA could be required by the embryo to produce such signalling molecules at specific stages of development. At pre-hatching stages, bovine embryos developed normally in a relatively high concentration of AA (100 µM). The lack of a developmental effect at a concentration significantly higher than that previously used in cultures of bovine embryos (~164 nM) (Lim and Hansel 1996; Lim and Hansel 2000), ovine placental cells (~1.6 µM) (Weems *et al.* 2003) or

mouse endometrial stromal cells (15 µM) (Zhao *et al.* 2012) strongly suggests that pre-hatching bovine embryos are largely irresponsive to AA. Nevertheless, we cannot rule out a biphasic effect of AA, which may mask a potential beneficial effect of AA at lower concentrations than 100 µM, which were not tested in this experiment as the main focus was on post-hatching development. Besides, the number of replicates and embryos analysed in the pre-hatching development experiment (Tables 2 and 3) was relatively low and maybe insufficient to identify a subtle developmental effect of AA supplementation at 100 µM on embryo development. The overtly unaltered pre-hatching development following AA supplementation at 100 µM may indicate a relatively lower ability of pre-hatching embryos to produce PGs, as no noticeable conceptus-induced changes in endometrium gene expression are observed before maternal recognition of pregnancy on Day 16 (Forde *et al.* 2011), and *PTGS2* – the rate limiting enzyme for the production of prostanoids (Simmons *et al.* 2004) – is sharply upregulated on Day 16 bovine conceptus (Mamo *et al.* 2011).

In contrast to pre-hatching embryos, post-hatching embryos were highly sensitive to AA: the same concentration (100 µM) at which no developmental effects were observed during pre-hatching embryo culture completely abrogated embryo development in the post-hatching culture, and a gradual reduction of embryonic size at Day 12 of *in vitro* culture was observed at 5–20 µM AA. This was an unexpected outcome, as a beneficial effect, particularly on the proliferation of extra-embryonic membranes (i.e. embryo size) was intended, given the expression of PGs receptors in the trophectoderm (Dorniak *et al.* 2011). A plausible explanation for the negative effect of AA addition at these stages is that the ability to metabolise AA could arise around blastocyst hatching but it may be too incipient to properly catalyse the conversion of AA into signalling molecules. *In vitro* Day 12 embryos

**Fig. 2.** Relative mRNA abundance in Day 12 embryos cultured at different concentrations of arachidonic acid. Different letters indicate significant differences based on ANOVA ( $P < 0.05$ ).

are developmentally equivalent to *in vivo* E10-11 embryos (Ramos-Ibeas *et al.* 2020), an earlier developmental stage than *in vivo* Day 12, when PGs production is well documented (Ulbrich *et al.* 2009). Further supporting this hypothesis, AA content in the embryo increases significantly from Day 7 to Day 14 (Menezo *et al.* 1982) and the ability of bovine embryos to metabolise AA changes substantially as conceptus elongation progresses (Lewis *et al.* 1982; Hwang *et al.* 1988). In particular, elongated conceptuses were found to metabolise AA into 13, 14-dihydro-15-keto-PGF2 $\alpha$ , PGF2 $\alpha$  and PGE2 at Days 16 and 19 of *in vivo* development, and PG production increased from Day 16 to Day 19 (Lewis *et al.* 1982). At earlier stages, *in vivo* embryos collected at Day 12 of the oestrous cycle metabolised AA primarily to PGE2, whereas those collected one day later metabolised AA to both PGE2 and PGF2 $\alpha$ , and embryos collected after Day 15 of the cycle metabolised AA to PGI in addition to PGE2 and PFG2 $\alpha$  (Hwang *et al.* 1988). Further improvements in the post-hatching system, such as those obtained in sheep embryos upon the addition of cytokines (Ramos-Ibeas *et al.* 2022), would serve to test if AA is beneficial at later stages of development, currently inaccessible by *in vitro* systems.

AA supplementation exerted a transcriptional regulation in Day 12 *in vitro*-derived embryos. Lactate dehydrogenase A (LDHA) and Stearoyl-CoA desaturase (SCD) transcription decreased gradually upon AA exposure. LDHA is a rate limiting enzyme of anaerobic glycolysis whose transcript abundance correlates with the activity of that metabolic pathway (Bermejo-Alvarez *et al.* 2010b). LDHA expression increases as embryo development progresses from Day 9 to Day 15 bovine *in vitro* development (Ramos-Ibeas *et al.* 2020), and from pre- to post-hatching porcine *in vivo* embryos (Liu *et al.* 2021), which suggests that its downregulation in response to AA may reflect a developmental delay caused by exposure to AA. SCD catalyses a rate limiting step in the synthesis of unsaturated fatty acids and, as occurred for LDHA, its expression increases progressively from the ovoid to the filamentous conceptus stage in bovines (Ribeiro *et al.* 2016b) and, therefore, its downregulation in AA exposed embryos may be also consequence of a developmental delay.

PPARG and PTGIS were downregulated in the presence of AA, showing a statistically significant downregulation in the group exposed to 5  $\mu$ M AA compared to the control. The downregulation of both PTGIS and PPARG are seemingly related, as PTGIS is the synthase of prostacyclin PGI2, and PGI2 is a known activator of PPARs (Desvergne and Wahli 1999). The downregulation of PTGIS and PPARG is consistent with a detrimental effect of AA addition to the post-hatching culture, as their mRNA levels increase along conceptus elongation (Cammass *et al.* 2006) and PGI2 may be required for post-hatching development, given that PGI2 supplementation during pre-hatching development has been reported to reduce apoptosis and enhance blastocyst expansion (Song *et al.* 2009). PPARG downregulation may be also associated with a developmental delay, as its transcription

increases progressively from the ovoid to the filamentous conceptus stage (Ribeiro *et al.* 2016b).

In conclusion, pre-hatching embryos are mostly irresponsive to AA, whereas a negative effect of AA was observed at early post-hatching development, suggesting that AA addition is not required to mimic *in vivo* conditions during early post-hatching development. This developmental delay was associated with downregulation of metabolism-related enzymes, a PG synthase and a lipid signalling receptor.

## Supplementary material

Supplementary material is available [online](#).

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**Data availability.** The data that support this study will be shared upon reasonable request to the corresponding author.

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