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Effect of α-linolenic acid on oocyte maturation and embryo development of prepubertal sheep oocytes

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ABSTRACT

The purpose of this study was to evaluate the effect of omega-3 α-linolenic acid (ALA) added to the IVM medium on embryo development of prepubertal sheep oocytes. Experiment 1 investigated the effect of ALA at different concentrations (0 [control], 50, 100, and 200 μM) and DMSO (100 μM) in IVM media on cumulus cell expansion and oocyte nuclear maturation and on synthesis of prostaglandins (PGE2 and PGF2α). Experiment 2 investigated the effects of ALA at different concentrations in the IVM medium on oocyte fertilization, cleavage, and developmental potential to blastocyst stage and changes in estradiol and progesterone concentrations in the spent IVM media. IVM oocytes were fertilized with frozen-thawed spermatozoa capacitated in a serum-free sperm medium. Presumptive zygotes were cultured 8 days in synthetic oviductal fluid (SOF) medium without serum. Blastocyst quality was assessed by counting total cell number and the number of apoptotic cells using Hoechst and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Nuclear maturation of oocytes and the number of fully expanded cumulus cells were reduced after treatment with 200 μM of ALA compared with other groups ($P \leq 0.05$). Supplementation with ALA increased both PGE2 and PGF2α concentrations in the spent media ($P \leq 0.05$). No differences were observed in blastocyst development among control (12.2%) and 50, 100, and 200 μM ALA groups (6.9%, 11.5% and 14.0%, respectively). However, the total cell number (46.50 ± 5.85, 67.94 ± 6.71, 45.20 ± 6.37, and 59.80 ± 5.51, respectively; $P \leq 0.05$) and apoptotic cell number (6.45 ± 0.89, 2.48 ± 0.81, 4.02 ± 1.15, and 3.67 ± 1.15, respectively; $P \leq 0.05$) were significantly improved. After IVM, estradiol concentration was lower and progesterone concentration was higher in ALA groups compared with the control group ($P \leq 0.05$). In conclusion, these results revealed that ALA affects prepubertal sheep embryo quality associated with alteration of releasing reproductive hormones.

1. Introduction

Reproduction in ruminants is associated with the accessibility of energy. Dietary fats support reproductive functions by supplying energy and by actions on reproductive performances. For example, increasing the amount of fatty acids (FAs) in diet increases steroid and eicosanoid secretion, which can alter ovarian and uterine functions and affect pregnancy rates (reviewed by [1]). The FA component of the diet also affects the oviductal and uterine environments (gene expression) to promote embryo development [2]. The animals fed with FAs have altered proportions of FAs in the follicular fluid [3] and show improved reproductive functions in bovine [4,5] and sheep [6,7]. Fatty acids are important sources of energy for oocytes and embryos [4]. Fouladi-Nashta et al. [8] reported

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that an increased level of rumen-inert FA in diet improved the developmental potential of bovine oocytes to the blastocyst stage and also embryo quality. However, no changes in the morphology of the oocytes (grades 1–4) or cleavage rate were found. But the blastocysts from the high-fat group contained considerably more total inner cell mass and trophectoderm cells compared with the low-fat group.

Polyunsaturated fatty acids (PUFAs) including omega-6 and omega-3 essential FAs are important for health but cannot be synthesized in the body and must be provided by diet (reviewed by [9]). They affect reproduction in both males and females. It has been shown that the PUFA content of oocytes might affect maturation, embryo development, and cryopreservation [10]. α-Linolenic acid (ALA) belongs to the omega-3 group (C18:3, n-3) and is involved in oocyte growth and differentiation and was reported to have a role in the regulation of meiotic arrest at the germinal vesicle (GV) stage (reviewed by [10]). In the body, ALA is transformed to eicosapentaenoic acid (EPA, C20:5).

The long-chain polyunsaturated FAs, EPA and ALA, are the precursors of eicosanoids including prostaglandins (PGs), which play important roles in reproduction functions such as ovulation, estrus, embryo survival, and parturition [11]. α-Linolenic acid is produced by the ovarian follicles and is present in the follicular fluid. It is involved in oocyte maturation and presumably in oocyte competence in development to the blastocyst stage (reviewed by [11]). Fatty acids may affect the maturation of oocytes by altering the structure of lipids in oocytes [8] or through changes in the type and concentrations of PGs or other metabolites present in the follicular fluid surrounding the oocyte [12]. Prostaglandin E2 is the dominant PG in the preovulatory follicles [13]. Several studies have reported that PGE2 plays important roles in cumulus cell expansion and oocyte maturation in rodents [14–17] and cattle [18]. Inhibition of PGE2 using PG-endoperoxide synthase 2 in mice decreases cumulus expansion and oocyte maturation [15]. Prostaglandin E2 is considered as a luteotrophic factor in the early luteal phase. Later in the cycle, PGF2α is the main luteolytic agent. It has been reported that ALA and EPA can inhibit PGF2α release in bovine endometrial cells (reviewed by [10]). The effects of ALA and its metabolite PGE on prepubertal sheep oocyte maturation and development are not clear.

Cumulus cells secrete the steroid hormones estradiol (E2) and progesterone (P4) [19]. Polyunsaturated fatty acids can influence ovarian steroid synthesis. For example, follicular E2 production was higher in cows supplemented with ALA [20], and some (but not all) studies have found low P4 concentrations in the early luteal phase in PUFA-treated animals (reviewed by [10]). Similarly, ALA increases P4 production in theca cells [19]. These actions are either mediated directly through the effects of PUFAs on steroid synthesis, e.g., through altered steroidogenic acute regulatory protein expression [21] or indirectly through altered PG production. The ratio of E2-to-P4 in follicular fluid has been used as a surrogate marker of healthy and atretic follicles [22].

The use of Juvenile *in vitro* Embryo Technology in breeding programmes is beneficial as it decreases the generation intervals and enhances the genetic gain. On the other hand, ovaries from prepubertal animals have higher numbers of oocytes than those from adult females [23]. However, *in vitro* embryo production from prepubertal animals is not as successful as adult females (reviewed by [24]). Thus, it has been shown that *in vitro* produced blastocysts are lower in prepubertal than in adult females (1.2 vs. 2.2 blastocysts per ovary) in cows [25]. Also the percentage of blastocysts obtained from IVM oocytes is lower in prepubertal ewes (20% vs. 40%) [26] and in goats (24% vs. 34%) [27]. Considering the beneficial effects of ALA supplementation in improving oocyte maturation and developmental potential, we have conducted experiments to assess whether supplementation of different ALA concentrations to prepubertal sheep oocyte IVM media can improve their fertilization rate and development to the blastocyst stage. We have also analyzed the concentrations of PGs and steroids in the spent media.

2. Materials and methods

2.1. Materials

Except otherwise mentioned, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Embryo culture media were incubated at 38.5 °C under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for at least 2 hours before culture. Washing and maturation media were incubated at 38.5 °C under a humidified atmosphere of 5% CO_2 in air for at least 2 hours before use for slicing and culture.

2.2. Oocyte collection

Ovaries from 3-month old prepubertal sheep were obtained from a local abattoir and brought to the laboratory within 1 hour in PBS at 30 °C to 35 °C. Cumulus oocyte complexes (COCs) were retrieved by slicing the ovaries in a 55-mm Petri dish containing washing media basically Tissue culture medium 199 supplemented with 20 mM of HEPES, and 4 mg/mL of FA-free BSA [28]. Afterward, the COCs were transferred into a 35-mm Petri dish and washed twice before moving to the maturation medium. The COCs with two layers or more of compact cumulus cells were selected for maturation.

2.3. In vitro maturation

A group of COCs was cultured (20 COCs for each treatment for nuclear maturation experiments and 45–50 for embryo development experiments) in 500 μL of maturation medium according to the experimental design. Maturation medium was prepared as previously reported by Shirazi et al. [29] with minor modifications. Tissue culture medium 199 was used as a basic media supplemented with 5 μg/mL of FSH (Follitropin; Bioniche Animal Health, Belleville, ON, Canada), 5 μg/mL of LH (Leutropin; Bioniche Animal Health), 1 μg/mL of estradiol 17 β (E2), 0.2 mM of sodium pyruvate, 2 mM of L-glutamine, 50 μg/mL of gentamicin, and 6 mg/mL of FA-free BSA. The COCs were incubated in four-well dishes

(NUNC; VWR International, Milan, Italy) for 24 hours at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

2.4. Oocyte maturation assessment: cumulus expansion and nuclear stage

Cumulus expansion was determined at 24 hours by subjected descriptions as not expanded, partially expanded, or fully expanded under a light microscope (Olympus SZH, DF plan 1X; Olympus). Oocyte nuclear stage in meiosis was determined after aceto-orcein staining [28]. Briefly, the oocytes were denuded by gentle pipetting and placed on a clean glass slide and overlaid with a square cover slip that was held up by four droplets of a vaseline-paraffin mixture (40:1). Afterward, they were fixed for at least 24 hours in glacial acetic acid (GAA) in methanol fixative solution (1:3). Thereafter, the oocytes were stained for 2 minutes with 1% orcein in a 45% GAA mixture before washing with a mixture of distilled water, glycerol, and GAA (3:1:1). Finally, the nuclear maturation was recorded under a phase contrast microscope (Olympus B201; Olympus).

2.5. Assessment of PGs by RIA

Concentrations of PGF2α and PGE2 in the spent maturation media were measured by RIA technique according to the Cheng et al. [28,30]. Briefly, the standards (range 0.05–10 ng/mL for PGE2 and 0.025–5 ng/mL for PGF2) or samples were mixed with anti-PGE2 and PGF2α serum (from Dr. N.L. Poyser; University of Edinburgh, Edinburgh, UK), and tritiated tracer ([5, 6, 8, 11, 12, 14, 15 (n)-^{3}H]-PGE2 and PGF2α; Amersham International PLC, Amersham [cat no. TRK431 and TRK464, respectively]) in duplicates. After 24 hours of incubation at 4 °C, dextran-coated charcoal suspension including 0.4% dextran (T-70; Amersham Pharmacia Biotech) and 2% neutralized charcoal were added to all tubes except the total count. They were incubated at 4 °C for 10 minutes and centrifuged at 2000× *g* for 10 minutes. The supernatant was removed into 6 mL scintillation vials containing 4 mL of scintillant (Ultima gold; Packard Bioscience BV, Pangbourne, Berks, UK) and counted for 2 minutes. The limit of detection was 2 pg/tube for PGE2 and 1 pg/tube for PGF2α. The intra-assay coefficients of variation were 3.5% and 4.1%, whereas the interassay coefficients were 6.3% and 9.6%, respectively.

2.6. In vitro fertilization

One hour before the end of the 24 hours of oocyte maturation period, the swim up method was commenced for preparation of semen for fertilization. For preparation of sperm capacitating media, the Tervit et al. [31] protocol was used with modifications that briefly included 107.70 mM of NaCl, 7.16 mM of KCl, 1.19 mM of KH_2PO_4, 0.49 mM of $MgCl_2$, 1.71 mM of $CaCl_2$, 3.3 mM of Na-lactate, 4 mM of $NaHCO_3$, 0.33 mM of Na-pyruvate, 0.103 mM of L-glutamine, 2.4 mM of D-glucose, 21 mM of HEPES, 20 μL/mL of penicillin-streptomycin, and 3 mg/mL of BSA fraction V. Swim up was performed according to the Shirazi et al. [29] method with some minor modifications in which straws were thawed in a 37 °C water bath for 30 seconds and the sperm contents were divided into four doses of 85 μL, which were added under 3 mL of sperm washing media in a 15 mL falcon tube. The tubes were placed at 45° angle in racks, which were placed in a 38.5 °C humidified incubator with 5% CO_2 in air for 45 minutes. Then the supernatants were removed and transferred to a new 15 mL falcon tube and centrifuged at 200× *g* for 7 minutes at room temperature. After centrifugation, the sperm pellet was resuspended in fertilization media containing 3.42 mM of $CaCl_2$, 9.9 mM of Na-lactate, 0.99 mM of Na-pyruvate, 1 mM of L-glutamine, 10 μL/mL of penicillin-streptomycin, and 2 mg/mL of BSA fraction V. Sperm concentration was determined by using the Neubauer counting chamber. *In vitro* fertilization was carried out by coincubating the matured COCs with 2 to 2.5 × 10^6 sperm per milliliter for 24 hours at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

2.7. Assessment of nuclear stage of zygotes

Twenty hours after fertilization, the presumptive zygotes were stained with 125 ng/mL of 4,6-diamidino-2-phenylindole (Vysis Inc., Downer's Grove, IL, USA) to assess fertilization rate by observing the presence of male and female pronuclei. Briefly, the presumptive zygotes were gently denuded by pipetting and fixed in 4% paraformaldehyde for 30 minutes and without washing they were mounted on a 4,6-diamidino-2-phenylindole droplet and overlaid with a cover slip. The pronucleus formation was recorded using a fluorescent microscope (Olympus B201, Olympus). The presence of male and female pronuclei was counted as a two-pronuclei (2PN) stage or normal fertilization. Polyspermy was oocytes with two or more sperm heads or more than two pronuclei, and asynchronous fertilization oocytes with the female pronucleus and a condensed sperm head.

2.8. In vitro embryo culture

After 20 hours of fertilization, presumptive zygotes were denuded from remaining cumulus cells by gentle pipetting and then washed in SOF media [31] containing 9.9 mM of Na-lactate, 0.99 mM of Na-pyruvate, 1 mM of L-glutamine, 2.24% basal medium eagle (BME) essential amino acids, 0.5% minimal essential medium (MEM) nonessential amino acids, 0.34 mM of trisodium citrate, 4.6 mM of myo-inositol, and 4 mg/mL BSA fraction V, three times in 100 μL drops. Thereafter, presumptive zygotes were transferred to 500 μL of SOF media and cultured until Day 8 after fertilization (fertilization Day = 0) at 38.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. The culture medium was refreshed every 48 hours (as nutrients in the medium could be depleted and also there is a possibility of accumulation of toxic products that can affect negatively further embryo development). At Day 8 (fertilization Day = 0) produced blastocysts were used to assess embryo quality.

2.9. Assessment of embryo quality

In vitro produced prepubertal sheep blastocysts were stained using Hoechst combined with TUNEL according to the Fouladi-Nashta et al. [32] protocol with some

modifications to count total cells and the number of apoptotic cells. Briefly, blastocysts were fixed in 4% paraformaldehyde containing 30 μg/mL of Hoechst for 10 minutes, washed three times in SOF-BSA media, and then permeabilized in 0.1% Triton 100X in SOF-BSA for 5 minutes. TUNEL staining using an fluorescein isothiocyanate (FITC)-conjugated *in situ* cell death detection kit (Roche, Penzberg, Germany) was performed according to the manufacturer's instructions without washing. The embryos were transferred into 10 μL droplets of a 1:10 dilution of the enzyme solution (terminal deoxynucleotidyl transferase from calf thymus) in dilution solution (nucleotide mixture) and incubated in a humid chamber for 1 hour at 38.5 °C. After incubation, the blastocysts were washed three times and mounted on 3 μL drops of glycerol based mounting media (Vectashield; Vector laboratories, Burlingame, CA, USA) and examined under an Olympus B201 fluorescent microscope (Olympus). The number of blue cells determined the total cells and the green cells as apoptotic cells.

2.10. Measurement of E2 and P4 by ELISA

The steroidogenic activity of cumulus cells was assessed in the maturation medium according to the procedure previously described by Maya-Soriano et al. [33]. Briefly, E2 and P4 concentrations were determined after 24 hours of IVM using commercial enzyme immunoassay kits (Estradiol ELISA Kit 402210 and Ultra Progesterone ELISA Kit 402410, respectively; Neogen Corporation, Lexington, USA) following the manufacturer's instructions. The assay was validated by determination of assay specificity (dilutional parallelism), accuracy from spike recovery (101.1% and 89.6% for E2 and P4 assays, respectively), precision from intra-assay variability (3.8% and 3.2% for E2 and P4 assays, respectively), and sensitivity (0.03 and 0.2 ng/mL for E2 and P4 assays, respectively).

2.11. Experimental design

Experiment 1 was done in three replicates with different concentrations of ALA (0 [control], 50, 100, and 200 μM) and 100 μM DMSO (was used for dissolving ALA) in IVM medium. After 24 hours of maturation, 303 COCs were used for evaluation of cumulus cell expansion and 286 COCs were denuded and used for nuclear division evaluation. The maturation medium was collected and frozen for measuring the PGE2 and PGF2α.

Experiment 2 was performed in four replicates to assess the effects of ALA concentrations (0 [control], 50, 100, and 200 μM) on IVF, oocyte cleavage, and blastocyst development and quality in the IVM medium. A total of 574 prepubertal sheep oocytes were fertilized and cultured *in vitro*. The DMSO group was not used in this experiment because according to experiment 1 we did not observe any effect of DMSO. The maturation medium was collected and frozen for measuring the E2 and P4.

2.12. Statistical analysis

Statistical analysis was carried out using IBM SPSS statistic for windows, version 20.0 (IBM Corp. Armonk, NY, USA). Linear mixed model was used for analyzing the number of total cells and apoptotic cells. The data for cumulus expansion, nuclear division (metaphase of the second meiotic division [MII]) is presented as the mean ± SEM, and two-pronulei (2PN), polyspermy status, cleavage, and blastocysts rate were analyzed by general linear model ANOVA and reported as a percentage ± SEM.

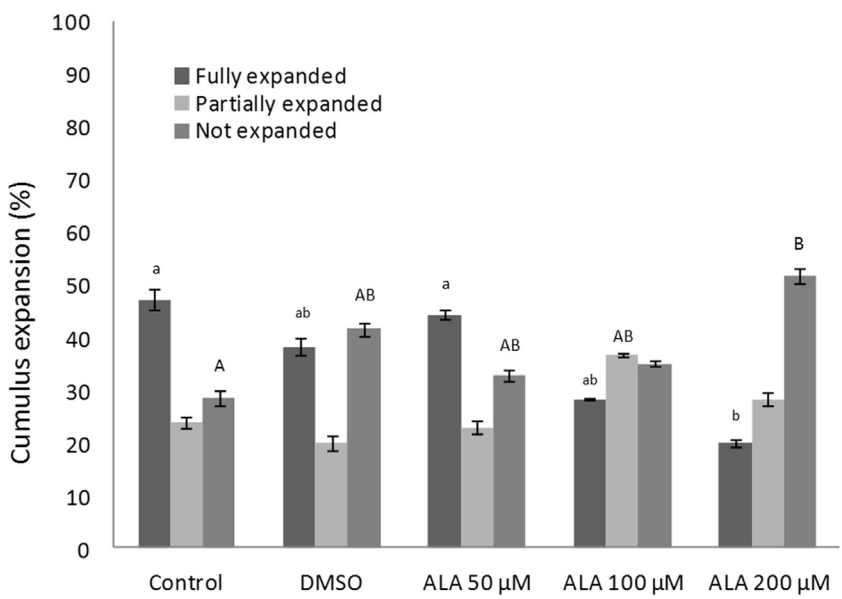

Fig. 1. Effect of ALA concentrations (50, 100, and 200 μM) added to IVM media on cumulus cell expansion of prepubertal sheep oocyte 24 hours after IVM. Data are presented as the mean ± SEM. Letters a, b indicate significant differences ($P \leq 0.05$) among experimental groups for fully expanded cumulus oocytes. Letters A, B indicate significant differences ($P \leq 0.05$) among experimental groups for not expanded cumulus oocytes. Partially expanded cumulus cell oocytes were not significantly different among experimental groups. ALA, α-linolenic acid; SEM, standard error of the mean.

Table 1
Effect of ALA concentrations (50, 100, and 200 μM) added to IVM media on nuclear stages of prepubertal sheep oocytes 24 hours after IVM.

Treatments	Total oocytes	GV, n (%)	GVBD, n (%)	MI, n (%)	AI, n (%)	TI, n (%)	MII, n (%)	Degenerated oocytes, n (%)
Control	58	3 (5.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	54 (93.1)[a]	1 (1.7)
DMSO	58	2 (3.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	54 (93.1)[a]	2 (3.4)
ALA 50 μM	58	2 (3.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	56 (96.6)[a]	0 (0.0)
ALA 100 μM	58	2 (3.4)	1 (1.7)	0 (0.0)	0 (0.0)	2 (3.4)	53 (91.4)[ab]	0 (0.0)
ALA 200 μM	52	2 (3.8)	0 (0.0)	0 (0.0)	0 (0.0)	4 (7.7)	43 (82.7)[b]	3 (5.8)

Data are presented as percentage, and different superscript letters (a, b) indicate significant differences among experimental groups ($P \leq 0.05$).
Abbreviations: A-I, anaphase of the first meiotic division; ALA, α-linolenic Acid; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metahase of the first meiotic division; MII, metaphase of the second meiotic division; TI, telophase of the first meiotic division.

The data for PGF2α and PGE2 were analyzed using ANOVA to compare the treatments of ALA with different concentrations (0 [control], 50, 100, and 200 μM). The data for PGF2α, PGE2, and the ratio of PGE2-to-PGF2α was transformed to $\log_{10}$ and E2, P4, and E2-to-P4 ratio to $\log_x$ before analysis.

3. Results

3.1. Experiment 1: effect of different ALA concentrations on oocyte maturation and PG secretions

The highest concentration (200 μM) of ALA significantly decreased the rate of fully expanded cumulus cells 24 hours after IVM compared with the control and 50 μM of ALA ($P \leq 0.05$; Fig. 1).

The results from oocyte maturation did not show differences on oocyte degeneration, GV, GV breakdown, MI (metaphase I), AI (anaphase I), and TI (telophase I) rates at 24 hours of IVM among experimental groups (Table 1). On the contrary, significantly lower rates of MII oocytes were found in the 200 μM ALA group compared with the control group (82.9% and 93.1%, respectively; $P \leq 0.05$; Table 1).

Figure 2 shows the concentration of PGE2 in the maturation media. After 24 hours of IVM, ALA treatment groups had significantly higher concentration of PGE2 than the control group ($P \leq 0.05$). No differences were observed between the control and DMSO groups. Figure 2 shows PGF2α concentration after IVM. The 50 and 100 μM ALA groups significantly increased PGF2α concentration compared with the control and DMSO groups ($P \leq 0.05$). No changes were observed in the PGF2α between the 200 μM ALA and control groups. Significant differences were observed in the ratio of PGE2-to-PGF2α between ALA groups (100 and 200 μM) and the control group ($P \leq 0.05$; Fig. 3).

3.2. Experiment 2: effects of different ALA concentrations on embryo development and synthesis of E2 and P4

In experiment 2, samples of IVF oocytes were stained to test the nuclear stage of the presumptive zygotes at 20 hours after fertilization. No differences were found in the total number of fertilized oocytes among the control and ALA groups. The percentage of normal zygotes (2PN) was statistically higher at 200 μM of ALA group than the control group (60.0% vs. 47.8%, respectively; $P \leq 0.05$). However, this difference was not observed between 50 and 100 μM ALA groups. The percentage of polyspermic oocytes was significantly ($P \leq 0.05$) higher in ALA groups (16%, 12%, and 5%, respectively; Table 2) than the control group (26%). There was no difference in total unfertilized oocytes

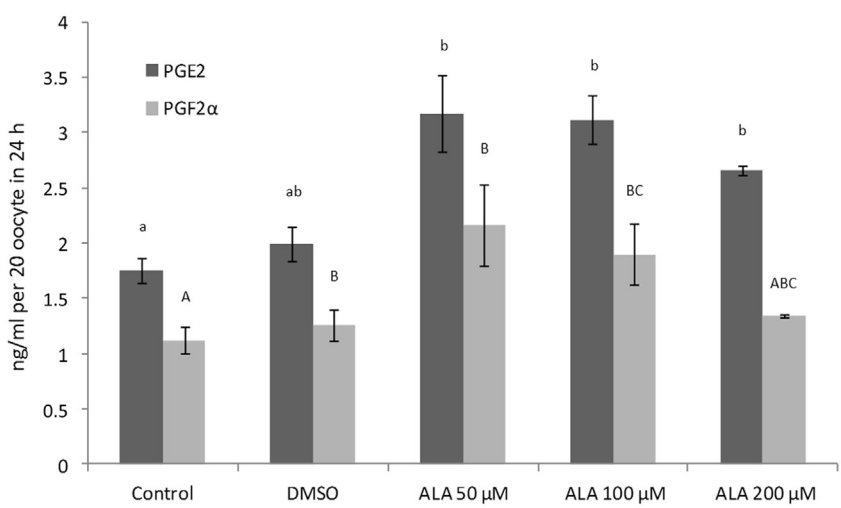

Fig. 2. Effect of ALA concentrations (50, 100, and 200 μM) added to IVM media on PGE2 and PGF2α synthesis. Results are presented as the mean ± SEM with $\log_{10}$. Letters a, b indicate significant differences for PGE2 concentrations among experimental groups ($P \leq 0.05$). Letters A, B, C indicate significant differences for PGF2α concentrations among experimental groups ($P \leq 0.05$). ALA, α-linolenic acid; SEM, standard error of the mean.

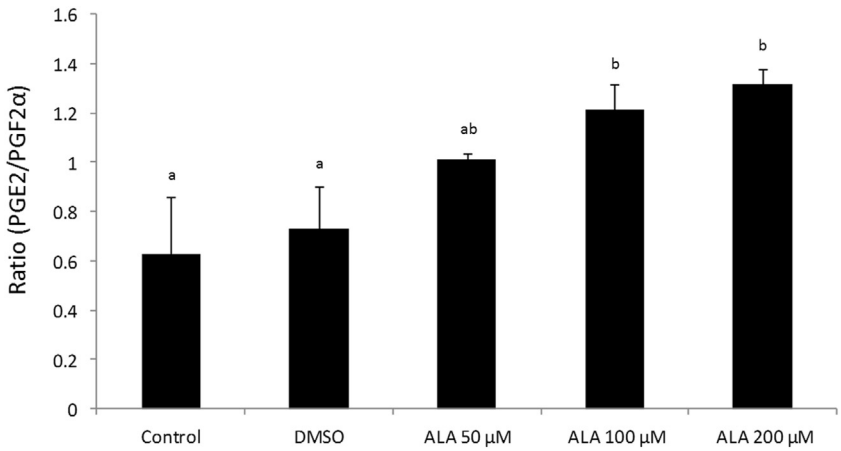

Fig. 3. Effect of ALA concentrations (50, 100, and 200 μM) added to IVM media on the ratio of PGE2-to-PGF2α. Results are presented as the mean ± SEM with $\log_{10}$. Different superscript letters indicate significant differences among experimental groups ($P \leq 0.05$). ALA, α-linolenic acid; SEM, standard error of the mean.

including degenerated and parthenogenetic oocytes among groups.

Table 3 shows the percentage of cleavage and embryo development at Days 6, 7, and 8 after fertilization (fertilization Day = 0). No differences were found among the ALA and control groups in cleavage and blastocyst development at Day 6, 7, and 8 after fertilization.

The quality of blastocysts 8 days after fertilization assessed by the number of total cells and the number of apoptotic cells is shown in Table 4. The total cell number in the control group was lower than the 50 and 200 μM ALA groups (46.5 vs. 67 and 59, respectively; $P \leq 0.05$). The number of apoptotic cells was significantly lower in 50 and 200 μM ALA groups compared with the control group (67.9, 58.8, and 46.5, respectively; $P \leq 0.05$; Table 4).

The concentration of E2 measured in the spent media was significantly lower in 50 and 200 μM ALA groups compared with the control group ($P \leq 0.05$; Fig. 4). However, 100 μM ALA group was not different compared with the control group. The concentration of P4 significantly increased after treatment with 100 μM of ALA compared with control group ($P \leq 0.05$; Fig. 5). This difference with the control group was not observed at 50 and 200 μM concentrations of ALA group (Fig. 5). Furthermore, the E2-to-P4 ratio was statistically lower at 50 and 100 μM of ALA group compared with the control group ($P \leq 0.05$; Fig. 6), but 200 μM ALA group did not show a significant change.

4. Discussion

Nuclear and cytoplasmic maturation are critical factors in determining developmental potential of oocytes after fertilization. Oocytes from prepubertal animals are often recovered from small follicles and are smaller in diameter [34]. These oocytes are deficient in the vital factors in the cytoplasm [24] and are not exposed to the hormonal microenvironment of ovulatory follicles. Provision of suitable IVM media could overcome some of these disadvantages and improve their developmental potential. Romaguera et al. [35] observed a similar percentage of blastocyst development between oocytes coming from follicles larger than 3 mm of prepubertal females and oocytes from adult goats. Likewise, Bender et al. [12] *via* analyzing follicular fluid composition found significantly higher ALA concentration in cow compared with heifer (29 vs. 14 μg/mL, respectively). Thus, addition of ALA to IVM media of prepubertal sheep oocytes could improve their embryo development. In cattle, the addition of 50 μM of ALA to IVM has improved oocyte nuclear maturation and embryo development [28]. This effect is mediated both directly through Mitogen-Activated Protein Kinase pathway and indirectly through PGE2 synthesis [28] and changing mitochondrial distribution and activity [36]. In the present study, we did not find differences in blastocyst production among the ALA-treated and control groups. However, the blastocyst quality, assessed by the number of total cells and apoptotic

Table 2
Effect of ALA concentrations (50, 100, and 200 μM) added to IVM media on IVF at 20 hours after insemination.

Experiments	Total oocytes	Unfertilized oocytes			Fertilized oocytes			
		Unfertilized, n (%)	Parthenogenetic, n (%)	Degenerated, n (%)	Fertilized oocytes, n (%)	2PN, n (%)	PS, n (%)	As, n (%)
Control	46	4 (8.69)	2 (4.34)	2 (4.34)	38 (82.60)	22 (47.82)^{a}	6 (13.04)^{a}	10 (26.3)^{a}
ALA 50 μM	41	2 (4.87)	0 (0.0)	2 (4.87)	37 (90.24)	19 (46.34)^{a}	12 (29.26)^{b}	6 (16.2)^{b}
ALA 100 μM	45	4 (8.88)	1 (2.22)	1 (2.22)	39 (86.66)	23 (51.11)^{a}	11 (24.44)^{b}	5 (12.8) ^{bc}
ALA 200 μM	45	5 (11.11)	1 (2.22)	1 (2.22)	38 (84.44)	27 (60.00)^{b}	9 (20.00)^{b}	2 (05.2)^{c}

Different letters (a, b) indicate significant differences among experimental groups ($P \leq 0.05$).
Abbreviations: AS, asynchronous fertilized oocyte; 2PN, oocytes with male and female pronucleus or normal fertilized zygote; PS, polyspermic fertilized oocytes.

Table 3
Effect of ALA concentrations (50, 100, and 200 μM) added to the IVM medium on cleavage and blastocyst development at 6, 7, and 8 days after insemination.

Experiments	Total oocytes	Total cleaved (%)	Two cells (%)	Four cells (%)	Eight cells (%)	Blastocyst/cleaved, n (%), Day 6	Blastocyst/cleaved, n (%), Day 7	Blastocyst/cleaved, n (%), Day 8
Control	139	108 (77.69)	5 (3.59)	28 (20.14)	75 (53.96)	3 (2.77)	9 (8.33)	17 (15.74)
ALA 50 μM	143	101 (70.62)	11 (7.69)	23 (16.08)	77 (53.84)	1 (0.99)	4 (3.96)	07 (6.93)
ALA 100 μM	149	122 (81.87)	9 (6.04)	24 (16.10)	89 (59.73)	2 (1.63)	7 (5.73)	14 (11.47)
ALA 200 μM	143	107 (74.82)	12 (8.39)	16 (11.18)	79 (55.24)	2 (1.86)	8 (7.47)	15 (14.01)

Abbreviation: ALA, α-linolenic acid.

cells, indicated an improvement in embryo matured with ALA supplementation.

Our results revealed that ALA at high concentration (200 μM) reduced the number of COCs with fully expanded cumulus cells. This was in agreement with the published results on maturation of bovine oocytes using this high ALA concentration [28]. There was also a reduction in the rate of oocytes reaching MII stage. These results confirmed that high concentrations of ALA could be harmful for prepubertal sheep oocytes too. Thus, it seems that 200 μM is an excessive concentration compared with physiological levels measured in sheep and bovine [6,37,38]. In cattle ALA levels of 0.04 mg/mL (143.66 μM) in plasma, 0.02 mg/mL (71.8 μM) in uterine endometrial tissue, and 0.02 mg/mL (71.8 μM) in follicular fluid were reported [37]. In sheep serum, n-3 high-density lipoprotein (HDL) concentration was 6.6 μM [6]. In our laboratory, we have observed ALA concentration of 40.8 and 22.7 μM in large (>3 mm) and small follicles, respectively, in prepubertal goats.

The results from the present study revealed that the addition of ALA to IVM media of prepubertal sheep oocytes increased PGE2 synthesis at a highly significant level in all ALA groups. A similar effect was reported in 50 μM ALA group in a bovine study [28]. Although, the main source of PGE2 production is the granulosa cells, also sheep and cattle COCs are able to synthesize it *in vitro* [39,40]. Similar findings were observed for prepubertal sheep in the studies presented here. In another study, researchers found that ALA at 100 μM significantly induced PGE2 synthesis in ovine amnion cells cultured *in vitro* [41]. Kirkup et al. [41] found that supplementation of 100 μM of PUFA (ALA, stearidonic acid, arachidonic acid, dihomo-γ-linoleic acid, and γ-linoleic acid) affected the type and quantity of PGs synthesized, thus arachidonic acid produced more two-series PGs and dihomo-γ-linoleic acid produced one-series PGs. The PGF2α is also important in several aspects of reproduction such as ovulation, estrus, embryo survival, and parturition [42]. In our study, 50 and 100 μM ALA concentrations significantly increased PGF2α secretion, but 200 μM was not statistically different compared with the control group. This reduction in PGF2α synthesis in this group could be associated with the low cumulus expansion and MII stage of oocytes matured with 200 μM of ALA. In conclusion, addition of ALA to the IVM medium of prepubertal sheep oocytes has increased the PGs synthesis, both PGE2 and PGF2α, but this increase has not affected oocyte nuclear maturation. The lack of positive effects of 50 and 100 μM of ALA on nuclear maturation, as it was observed in cattle [28], could be because of the specific characteristics of oocytes coming from ovaries of prepubertal lambs. The highest ALA concentration, 200 μM, has negatively affected nuclear maturation and cumulus expansion as it was observed in cattle.

Table 4
Effect of ALA concentrations (0 [control], 50, 100, and 200 μM) added to the IVM medium on blastocyst quality assessed by the number of total cells and apoptotic cells.

Treatments	Total blastocyst	Total cells	Apoptotic cells
Control	9	46.50 ± 5.85^{A}	6.45 ± 0.89^{a}
ALA 50 μM	5	67.94 ± 6.71^{B}	2.48 ± 0.81^{b}
ALA 100 μM	6	45.20 ± 6.37^{A}	4.02 ± 1.15^{a}
ALA 200 μM	10	59.80 ± 5.51^{B}	3.67 ± 1.15^{b}

Results are expressed as the mean ± SEM for treatments. Letters (A, B) indicate significant differences in total cells among experimental groups. Letters (a, b) indicate significant differences in apoptotic cells among experimental groups ($P \leq 0.05$).
Abbreviations: ALA, α-linolenic acid; SEM, standard error of the mean.

In experiment 2, we examined the effect of addition of ALA to IVM media on IVF and embryo development of prepubertal sheep oocytes. α-Linolenic acid concentrations had no effect on the total fertilized oocytes; however, 200 μM of ALA increased the number of normal zygotes (2PN) compared with the control group. The rate of polyspermic zygotes was significantly higher in all ALA concentrations compared with the control group. Interestingly, the percentage of asynchronous zygotes (one female pronucleus and one condensed sperm head) was significantly lower in ALA treatments compared with the control group. This could be considered as an improvement of male pronucleus formation under ALA treatments, whereas there was no difference in total fertilization rate between ALA treatments and the control group. Similarly, Childs et al. [43] have shown no effect of palmitic acid and n-3 PUFA used in diet on embryo yield and quality in heifer beef cows [43]. In contrast, Marei et al. [28] observed positive effects using ALA in IVM of bovine oocytes on embryo development. Likewise, Hughes et al. [7] using n-3 PUFA-enriched serum (5%, v:v) in the SOF culture medium observed an increase in blastocyst production, increased transcript expression for the antioxidant enzyme superoxide dismutase 1, but there was also an increase in morphologically poor embryos. Our results in prepubertal sheep oocytes revealed that there are no significant differences in cleavage and blastocyst rates among the control and ALA experimental groups (Table 3). This is in agreement with a report from Wonnacott et al. [6] who did not find any improvement in cleavage and blastocyst rates when adding omega-3 or omega-6 HDL to IVM media of sheep oocytes. Marei et al. [28] supplementing 50 μM of ALA to the IVM medium of bovine oocytes have shown an increase in cleavage, blastocyst, and hatched blastocyst rates. Similar

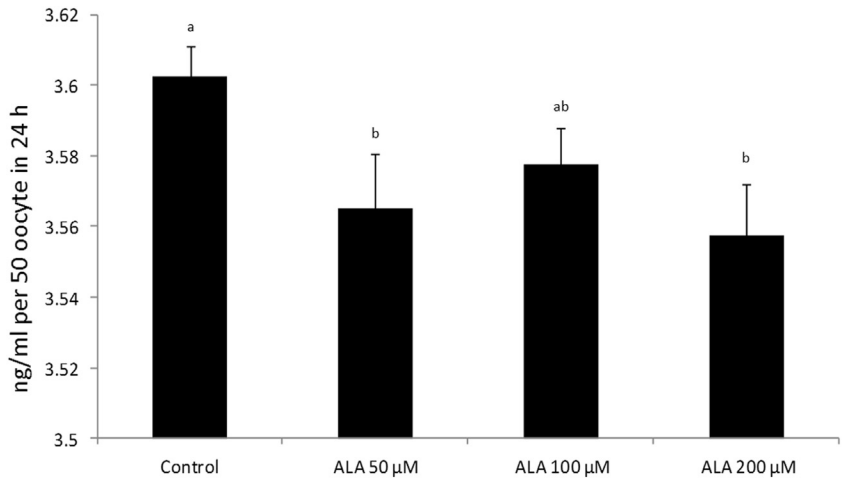

Fig. 4. Effect of ALA concentrations (50, 100, and 200 μM) added to the IVM medium on estradiol concentration in the spend maturation media. Results are presented as the mean ± SEM with $\log_x$. Different superscript letters indicate significant differences among experimental groups ($P \leq 0.05$). ALA, α-linolenic acid; SEM, standard error of the mean.

to this report, we have observed better quality blastocysts produced from oocytes matured in the presence of 50 μM of ALA and assessed by the number of total cells and apoptotic cells (Table 4). In conclusion, ALA has improved male pronuclear formation in zygotes and blastocyst quality, which could reflect changes in the cytoplasmic maturation of prepubertal sheep oocytes and needs further investigation.

After 24 hours of IVM, the concentration of E2 was lower and the concentration of P4 was higher in the maturation of ALA groups compared with the control group. Hughes et al. [7] found that using n-3 PUFA increases the synthesis of P4 by ovine theca cells. Also, Bao et al. [44] reported that HDL enhanced P4 production. Likewise, Wonnacott et al. [6] observed a high P4 and low E2 synthesis from granulosa cells cultured *in vitro* for 96 hours coming from small and medium size follicles of sheep ovaries fed with n-3 and n-6 HDL. It has been described that bovine healthy follicles have higher E2 level compared with atretic follicles, and atretic follicles have substantially elevated P4 level or theca products such as testosterone or androstenedione for the same size of follicle (reviewed by [45]). In rhesus monkey, Zheng et al. [46] have shown that the absence of E2 and P4 in IVM caused a failure of oocytes to develop to the blastocyst stage. In our study, we continued the maturation period to assess embryo developmental competence subsequent to ALA supplementation and the results revealed that embryo development was not affected by the level of steroid hormones during IVM (Figs. 4 and 5). Silva and Knight [47] concluded that the addition of P4 to IVM of bovine oocytes reduced the proportion of embryos forming blastocysts. The increased P4 concentration under ALA

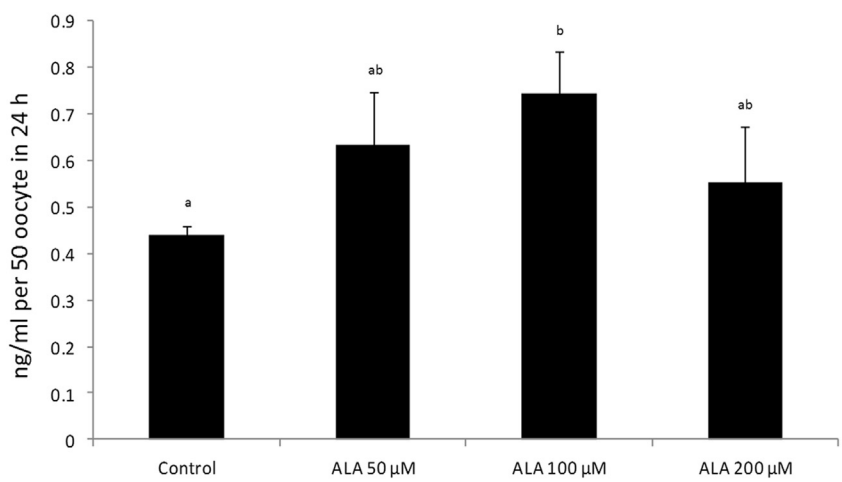

Fig. 5. Effect of ALA concentrations (50, 100, and 200 μM) added to the IVM medium on progesterone concentration in the spend maturation media. Results are presented as the mean ± SEM with $\log_x$. Different superscript letters indicate significant differences among experimental groups ($P \leq 0.05$). ALA, α-linolenic acid; SEM, standard error of the mean.

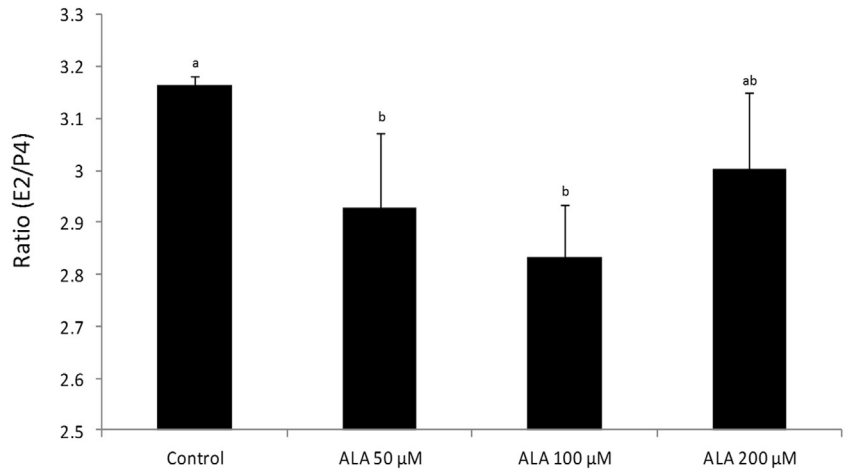

Fig. 6. Effect of ALA concentrations (50, 100, and 200 μM) added to the IVM medium on the ratio of E2-to-P4 concentration in the spend maturation media. Results are presented as the mean ± SEM with $\log_x$. Different superscript letters indicate significant differences among experimental groups ($P \leq 0.05$). ALA, α-linolenic acid; SEM, standard error of the mean.

supplementation of maturation media might be because of increased mRNA and protein expression of steroid acute regulator in the cumulus cells [10] or differentiation-luteinization of cumulus cells after 24 hours of culture (reviewed by [48]). The increase of P4 in the maturation medium had a negative effect on oocyte developmental potential leading to the production of poor quality embryos [10]. As the oocytes used in these experiments were derived from follicles smaller than 3 mm, COCs collected from small follicles in prepubertal ewes have machinery to produce both steroids and respond to gonadotrophic hormones FSH and LH present in the maturation medium to produce more P4 compared with E2. The higher level of P4 in the maturation medium affected maturation of oocytes and may have contributed to the low blastocyst rate and quality. However, it has recently been shown that oocyte competence leading to the blastocyst stage is not related to E2 and P4 concentrations in the follicular fluid [49].

It has been shown that lower E2 levels and higher P4-to-E2 ratios were associated with fast-cleaving embryos [50], which in other words mean that higher 2PN in ALA treatments could be related to the lower E2-to-P4 ratio in this study. However, in our study, the high concentration of P4 in ALA groups did not have a negative effect on blastocyst yield or quality.

The percentage of blastocyst-oocyte found in our study in the control group was 12.23%. In another study, from our group, Catalá et al. [51] using serum in each step of the *in vitro* embryo production protocol found that the percentage of blastocysts obtained from prepubertal ewes was 13.6% and 13.1% in IVF and intracytoplasmic sperm injection system, respectively. Also, O'Brien et al. [52] observed a 15.4% blastocyst rate. The total cell number of blastocysts in the present study was 46.50 ± 5.8, whereas Catalá et al. [51] observed blastocysts with higher number of cells (56.8 ± 4.8 in the IVF system and 62.7 ± 7.8 in the intracytoplasmic sperm injection system). Therefore, the lower number of cells found in the present study in blastocysts at 8 days after insemination could be because of serum omission in our protocols that were entirely serum-free for all experiments. Rizos et al. [53] have shown that using serum in IVM and IVC media bovine embryo development affects the speed of embryo development and quality of the forming blastocysts. Also, Lonergan et al. [54] have found that using fetal calf serum in after insemination culture media for embryo development affects the incidence and severity of mixoploidy in the achieved blastocyst.

4.1. Conclusions

Evidence from the present study revealed that the addition of 200 μM of ALA to the IVM medium of prepubertal sheep oocytes had a negative effect on nuclear maturation and cumulus cell expansion. This negative effect was not observed at 50 and 100 μM ALA concentrations. Oocytes matured with ALA improved male pronucleus formation and blastocyst quality; however, no effects were found on cleavage and blastocyst rates. The concentration of PGE2 and PGF2α in the IVM media was higher in ALA groups compared with the control group. E2 concentration was reduced and P4 concentration increased in ALA groups compared with control. In our entirely serum-free medium, the results on blastocyst production were similar to other research groups but the lack of serum has affected the number of total cells of the blastocysts.

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References

[1] Mattos R, Staples C, Thatcher W. Effects of dietary fatty acids on reproduction in ruminants. Rev Reprod 2000;5:38–45.
[2] Bilby T, Block J, Do Amaral B, Sa Filho O, Silvestre F, Hansen P, et al. Effects of dietary unsaturated fatty acids on oocyte quality and

follicular development in lactating dairy cows in summer. J Dairy Sci 2006;89:3891–903.
[3] Homa S, Brown C. Changes in linoleic acid during follicular development and inhibition of spontaneous breakdown of germinal vesicles in cumulus-free bovine oocytes. J Reprod Fertil 1992;94:153–60.
[4] Sturmey R, Reis A, Leese H, McEvoy T. Role of fatty acids in energy provision during oocyte maturation and early embryo development. Reprod Domest Anim 2009;44:50–8.
[5] Fouladi-Nashta AA, Wonnacott KE, Gutierrez CG, Gong JG, Sinclair KD, Garnsworthy PC, et al. Oocyte quality in lactating dairy cows fed on high levels of n-3 and n-6 fatty acids. Reproduction 2009;138:771–81.
[6] Wonnacott KE, Kwong WY, Hughes J, Salter AM, Lea RG, Garnsworthy PC, et al. Dietary omega-3 and -6 polyunsaturated fatty acids affect the composition and development of sheep granulosa cells, oocytes and embryos. Reproduction 2010;139:57–69.
[7] Hughes J, Kwong WY, Li D, Salter AM, Lea RG, Sinclair KD. Effects of omega-3 and -6 polyunsaturated fatty acids on ovine follicular cell steroidogenesis, embryo development and molecular markers of fatty acid metabolism. Reproduction 2011;141:105–18.
[8] Fouladi-Nashta AA, Gutierrez CG, Gong JG, Garnsworthy PC, Webb R. Impact of dietary fatty acids on oocyte quality and development in lactating dairy cows. Biol Reprod 2007;77:9–17.
[9] Spector AA. Essentiality of fatty acids. Lipids 1999;34:S1–3.
[10] Wathes DC, Abayasekara DR, Aitken RJ. Polyunsaturated fatty acids in male and female reproduction. Biol Reprod 2007;77:190–201.
[11] Gulliver CE, Friend MA, King BJ, Clayton EH. The role of omega-3 polyunsaturated fatty acids in reproduction of sheep and cattle. Anim Reprod Sci 2012;131:9–22.
[12] Bender K, Walsh S, Evans AC, Fair T, Brennan L. Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows. Reproduction 2010;139:1047–55.
[13] Segi E, Haraguchi K, Sugimoto Y, Tsuji M, Tsunekawa H, Tamba S, et al. Expression of messenger RNA for prostaglandin E receptor subtypes EP4/EP2 and cyclooxygenase isozymes in mouse periovulatory follicles and oviducts during superovulation. Biol Reprod 2003;68:804–11.
[14] Eppig JJ. Prostaglandin E2 stimulates cumulus expansion and hyaluronic acid synthesis by cumuli oophori isolated from mice. Biol Reprod 1981;25:191–5.
[15] Hizaki H, Segi E, Sugimoto Y, Hirose M, Saji T, Ushikubi F, et al. Abortive expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP(2). Proc Natl Acad Sci U S A 1999;96:10501–6.
[16] Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, et al. Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 1997;91:197–208.
[17] Takahashi T, Morrow JD, Wang H, Dey SK. Cyclooxygenase-2-derived prostaglandin E(2) directs oocyte maturation by differentially influencing multiple signaling pathways. J Biol Chem 2006; 281:37117–29.
[18] Nuttinck F, Gall L, Ruffini S, Laffont L, Clement L, Reinaud P, et al. PTGS2-related PGE2 affects oocyte MAPK phosphorylation and meiosis progression in cattle: late effects on early embryonic development. Biol Reprod 2011;84:1248–57.
[19] Vanderhyden BC, Cohen JN, Morley P. Mouse oocytes regulate granulosa cell steroidogenesis. Endocrinology 1993;133:423–6.
[20] Robinson R, Pushpakumara P, Cheng Z, Peters A, Abayasekara D, Wathes D. Effects of dietary polyunsaturated fatty acids on ovarian and uterine function in lactating dairy cows. Reproduction 2002; 124:119–31.
[21] Wang X, Walsh LP, Reinhart AJ, Stocco DM. The role of arachidonic acid in steroidogenesis and steroidogenic acute regulatory (StAR) gene and protein expression. J Biol Chem 2000;275:20204–9.
[22] Modina SC, Tessaro I, Lodde V, Franciosi F, Corbani D, Luciano AM. Reductions in the number of mid-sized antral follicles are associated with markers of premature ovarian senescence in dairy cows. Reprod Fertil Dev 2014;26:235–44.
[23] Koeman J, Keefer CL, Baldassarre H, Downey BR. Developmental competence of prepubertal and adult goat oocytes cultured in semi-defined media following laparoscopic recovery. Theriogenology 2003;60:879–89.
[24] Armstrong D. Effects of maternal age on oocyte developmental competence. Theriogenology 2001;55:1303–22.
[25] Palma G, Clement-Sengewald A, Krefft H. In vitro production of cattle embryos from calf oocytes. Theriogenology 1993;39:278.
[26] Ledda S, Bogliolo L, Calvia P, Leoni G, Naitana S. Meiotic progression and developmental competence of oocytes collected from juvenile and adult ewes. J Reprod Fertil 1997;109:73–8.
[27] Leoni GG, Succu S, Satta V, Paolo M, Bogliolo L, Bebbere D, et al. In vitro production and cryotolerance of prepubertal and adult goat blastocysts obtained from oocytes collected by laparoscopic oocyte-pick-up (LOPU) after FSH treatment. Reprod Fertil Dev 2009;21: 901–8.
[28] Marei WF, Wathes DC, Fouladi-Nashta AA. The effect of linolenic acid on bovine oocyte maturation and development. Biol Reprod 2009;81:1064–72.
[29] Shirazi A, Ardali MA, Ahmadi E, Nazari H, Mamuee M, Heidari B. The effect of macromolecule source and type of media during in vitro maturation of sheep oocytes on subsequent embryo development. J Reprod Infertil 2012;13:13–20.
[30] Cheng Z, Robinson R, Pushpakumara P, Mansbridge R, Wathes D. Effect of dietary polyunsaturated fatty acids on uterine prostaglandin synthesis in the cow. J Endocrinol 2001;171:463–73.
[31] Tervit H, Whittingham D, Rowson L. Successful culture in vitro of sheep and cattle ova. J Reprod Fertil 1972;30:493–7.
[32] Fouladi-Nashta A, Alberio R, Kafi M, Nicholas B, Campbell K, Webb R. Differential staining combined with TUNEL labelling to detect apoptosis in preimplantation bovine embryos. Reprod Biomed Online 2005;10:497–502.
[33] Maya-Soriano M, López-Gatius F, Andreu-Vázquez C, López-Béjar M. Bovine oocytes show a higher tolerance to heat shock in the warm compared with the cold season of the year. Theriogenology 2013; 79:299–305.
[34] Gandolfi F, Brevini T, Cillo F, Antonini S. Cellular and molecular mechanisms regulating oocyte quality and the relevance for farm animal reproductive efficiency. Rev Sci Tech 2005;24:413.
[35] Romaguera R, Moll X, Morató R, Roura M, Palomo MJ, Catalá MG, et al. Prepubertal goat oocytes from large follicles result in similar blastocyst production and embryo ploidy than those from adult goats. Theriogenology 2011;76:1–11.
[36] Marei WF, Wathes DC, Fouladi-Nashta AA. Impact of linoleic acid on bovine oocyte maturation and embryo development. Reproduction 2010;139:979–88.
[37] Childs S, Hennessy A, Sreenan J, Wathes D, Cheng Z, Stanton C, et al. Effect of level of dietary n-3 polyunsaturated fatty acid supplementation on systemic and tissue fatty acid concentrations and on selected reproductive variables in cattle. Theriogenology 2008;70: 595–611.
[38] Adamiak S, Powell K, Rooke J, Webb R, Sinclair K. Body composition, dietary carbohydrates and fatty acids determine post-fertilisation development of bovine oocytes in vitro. Reproduction 2006;131: 247–58.
[39] Marei WF, Abayasekara DRE, Wathes DC, Fouladi-Nashta AA. Role of PTGS2-generated PGE2 during gonadotrophin-induced bovine oocyte maturation and cumulus cell expansion. Reprod Biomed Online 2014;28:388–400.
[40] Fitz T, Hoyer P, Niswender G. Interactions of prostaglandins with subpopulations of ovine luteal cells. I. Stimulatory effects of prostaglandins E1, E2 and I21. Prostaglandins 1984;28:119–26.
[41] Kirkup SE, Cheng Z, Elmes M, Wathes DC, Abayasekara DRE. Polyunsaturated fatty acids modulate prostaglandin synthesis by ovine amnion cells in vitro. Reproduction 2010;140:943–51.
[42] Abayasekara D, Wathes D. Effects of altering dietary fatty acid composition on prostaglandin synthesis and fertility. Prostaglandins Leukot Essent Fatty Acids 1999;61:275–87.
[43] Childs S, Carter F, Lynch CO, Sreenan JM, Lonergan P, Hennessy AA, et al. Embryo yield and quality following dietary supplementation of beef heifers with n-3 polyunsaturated fatty acids (PUFA). Theriogenology 2008;70:992–1003.
[44] Bao B, Thomas M, Griffith M, Burghardt R, Williams G. Steroidogenic activity, insulin-like growth factor-I production, and proliferation of granulosa and theca cells obtained from dominant preovulatory and nonovulatory follicles during the bovine estrous cycle: effects of low-density and high-density lipoproteins. Biol Reprod 1995;53: 1271–9.
[45] Rodgers RJ, Irving-Rodgers HF. Morphological classification of bovine ovarian follicles. Reproduction 2010;139:309–18.
[46] Zheng P, Si W, Bavister BD, Yang J, Ding C, Ji W. 17β-Estradiol and progesterone improve in-vitro cytoplasmic maturation of oocytes from unstimulated prepubertal and adult rhesus monkeys. Hum Reprod 2003;18:2137–44.
[47] Silva C, Knight P. Effects of androgens, progesterone and their antagonists on the developmental competence of in vitro matured bovine oocytes. J Reprod Fertil 2000;119:261–9.
[48] Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. Minireview: functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Mol Reprod Dev 2002;61:414–24.

[49] Matoba S, Bender K, Fahey AG, Mamo S, Brennan L, Lonergan P, et al. Predictive value of bovine follicular components as markers of oocyte developmental potential. Reprod Fertil Dev 2014;26:337–45.
[50] Morgan PM, Boatman DE, Bavister BD. Relationships between follicular fluid steroid hormone concentrations, oocyte maturity, in vitro fertilization and embryonic development in the rhesus monkey. Mol Reprod Dev 1990;27:145–51.
[51] Catalá MG, Izquierdo D, Rodríguez-Prado M, Hammami S, Paramio M-T. Effect of oocyte quality on blastocyst development after in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) in a sheep model. Fertil Steril 2012;97:1004–8.
[52] O'Brien JK, Catt SL, Ireland KA, Maxwell WMC, Evans G. In vitro and in vivo developmental capacity of oocytes from prepubertal and adult sheep. Theriogenology 1997;47:1433–43.
[53] Rizos D, Gutierrez-Adan A, Perez-Garnelo S, De La Fuente J, Boland M, Lonergan P. Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. Biol Reprod 2003;68:236–43.
[54] Lonergan P, Pedersen HG, Rizos D, Greve T, Thomsen PD, Fair T, et al. Effect of the post-fertilization culture environment on the incidence of chromosome aberrations in bovine blastocysts. Biol Reprod 2004; 71:1096–100.