Effect of Linolenic acid during in vitro maturation of ovine oocytes: embryonic developmental potential and mRNA abundances of genes involved in apoptosis

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Abstract

Purpose To study the effect of α -linolenic acid (ALA) on meiotic maturation, mRNA abundance of apoptosis-related (*Bax* and *Bcl-2*) molecules, and blastocyst formation in ovine oocytes. **Methods** A preliminary experiment was conducted to analyze the concentration of ALA in “small” (≤ 2 mm) and “large” (≥ 6 mm) follicles using gas chromatography/mass spectrometry analysis. The concentration of ALA in small and large follicles was determined to be in a range of 75.4 to 125.7 μ M,

Capsule α -Linolenic acid in ovine oocyte maturation medium has been found to affect developmental competence, and expression of the *Bax*, and *Bcl-2* genes

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respectively. In vitro maturation (IVM) of oocyte was then performed in presence of 0 (control), 10 (ALA-10), 50 (ALA-50), 100 (ALA-100), and 200 (ALA-200) μ M of ALA. Meiotic maturation and mRNA abundance of *Bax*, and *Bcl-2* genes was evaluated after 24 h of IVM. The embryonic cleavage and blastocyst formation following parthenogenetic activation were also determined for each group.

Results The highest concentration of ALA (ALA-200) decreased the oocyte maturation rate compared with the control group. Analysis of apoptosis-related genes in oocytes after IVM revealed lesser transcript abundances for *Bax* gene, and higher transcript abundances for *Bcl-2* gene in ALA-treated oocytes as compared with the control oocytes. In term of cleavage rate (considered as 2-cell progression), we did not observe any differences among the groups. However, ALA-100 group promoted more blastocyst formation as compared with the control group.

Conclusion Our results suggested that ALA treatment during IVM had a beneficial effect on developmental competence of ovine oocytes by increasing the blastocyst formation and this might be due to the altered abundance of apoptosis-regulatory genes.

Keywords α -linolenic acid · Ovine oocyte · Apoptosis genes · Blastocyst formation

Introduction

The lipid profile of the oocyte is dynamic and has been shown to be largely dictated by the environment in which it develops [1]. Previous reports have shown that supplementary fatty acids have significant effects on oocyte maturation and subsequent pre-implantation development [2–5].

Lipid analysis of follicular fluid revealed that ω -6 and ω -3 fatty acid families are the most abundant polyunsaturated fatty acids (PUFAs) in the follicle [6]. These findings strengthen the hypothesis that dietary supplementation of ω -6 and/or ω -3 fatty acids has an important role in oocyte quality and development [7]. Therefore, dietary supplementation of heifers with high levels of fish oil, which is a rich source of n-3 fatty acids, significantly increased the total amount of n-3 PUFAs and the n-3/n-6 ratio in follicular fluid [8]. In the former study, alteration of PUFAs through the dietary sources of FAs led to improvement of oocyte development potential [7]. In bovine species, supplementation of maturation medium with essential fatty acids has been shown to have positive [3] or negative [4] effects on oocyte developmental competence depending on the type of fatty acid studied.

Although numerous studies have advanced knowledge of the influence of fat and mix of fatty acids on oocyte developmental competence [9–13], data on the effect of each family or each specific fatty acid are rare and the results of the existing studies are somewhat discordant. All-*cis*-9,12,15-octadecatrienoic acid or α -linolenic acid (ALA, 18:3 n-3, an essential fatty acid of the ω -3 family) is a PUFA highly abundant in some vegetable oils and sea fishes [14]. There have been only a few reports about the effect of α -linolenic acid on oocyte developmental competence, particularly in domestic animals [15].

In order to further investigate the hypothesis that developmental competence of in vitro matured oocytes can be influenced by exogenous ALA, a prospective study was conducted in a set of four experiments. Initially, a preliminary experiment was performed to analyze the level of ALA in “small” (≤ 2 mm) and “large” (≥ 6 mm) follicles. Thereafter, the effect of different concentrations of ALA (estimated from preliminary data) on oocyte meiotic maturation was studied. Apoptosis status of oocytes was also assessed by analyzing mRNA abundance of *Bax*, and *Bcl-2* genes after maturation in vitro. Furthermore, the effect of exogenous ALA on the developmental competence of blastocyst formation was examined after parthenogenetic stimulation.

Materials and methods

All chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and Gibco (Grand Island, NY, USA) unless otherwise stated.

Collection of ovaries

Ovaries were collected from adult ewes at a local slaughterhouse and transported to the laboratory within 1–2 h in thermos flask containing 0.9 % saline (32–37 °C).

Aspiration of follicular fluid

Upon arrival, ovaries were washed in pre-warmed fresh saline. Visible follicles were measured and grouped according to diameters as “small” (≤ 2 mm) and “large” (≥ 6 mm). Follicular fluid (FF) was aspirated using 5 ml syringe (18G needles) and pooled within each group. After centrifugation at $2400 \times g$ for 5 min at 4 °C, the supernatant fluid was aspirated and stored at –20 °C until analysis.

Gas chromatography–mass spectrometry

Gas chromatography–mass spectrometry (GC-MS) analysis was performed to determine the fatty acid composition of FF. For this, fatty acid methyl ester (FAME) synthesis was performed according to the previously described methods by O’Fallon [16]. Briefly, FF samples were thawed on ice prior to analysis. After thawing, 1 ml of FF sample was supplemented with 1 ml of C13:0 as an internal standard (0.5 mg of C13:0/ml of MeOH), 0.7 ml of 10 mol/l KOH in dH₂O, and 5.3 ml of MeOH. The tube was incubated in a 55 °C water bath for 1.5 h with vigorous hand-shaking every 20 min. After cooling below room temperature, 0.58 ml of 12 mol/l of H₂SO₄ in dH₂O was added. The tube content was mixed by inversion, and with precipitated K₂SO₄ present incubated again in a 55 °C water bath for 1.5 h with hand-shaking every 20 min. After FAME synthesis, the tube was cooled and 3 ml of hexane was added; the tube was vortex-mixed for 5 min and centrifuged for 5 min, and the hexane layer, containing the FAME, was placed into a GC vial.

The fatty acid composition of the FAME was determined by capillary GC on a SP-2560, 100 m $\times$ 0.25 mm $\times$ 0.20 μ m capillary column (Supelco) installed on a Hewlett Packard 5890 gas chromatograph equipped with a Hewlett Packard 3396 Series II integrator and 7673 controller, a flame ionization detector, and split injection (Agilent Technologies Inc., Santa Clara, CA). After injection of sample to GC the initial oven temperature was 140 °C, held for 5 min, subsequently increased to 240 °C at a rate of 4 °C/min, and then held for 20 min. Helium was used as the carrier gas at a flow rate of 0.5 ml/min, and the column head pressure was 280 kPa. Both the injector and the detector were set at 260 °C. The split ratio was 30:1. Fatty acids were identified by comparing their retention times with the fatty acid methyl standards [16].

Oocyte collection

Cumulus-oocyte complexes (COCs) were isolated from antral follicles using the slicing method. Only oocytes completely surrounded by compact and thick cumulus and homogenous cytoplasm were randomly selected and used for the experiments.

In vitro maturation (IVM)

COCs were washed three times in maturation medium consisting of bicarbonate-buffered TCM-199 with 2 mM L-glutamine supplemented with 10 % fetal bovine serum, 5.5 mg mL⁻¹ sodium pyruvate, 25 µg mL⁻¹ gentamycin sulphate, 5.0 µg mL⁻¹ LH, 0.5 µg mL⁻¹ FSH, and 1 µg mL⁻¹ estradiol. A group of 10 COCs were cultured per 50 µl drop of maturation medium in 30 mm petri dish for 24 h at 38.5 °C in 5 % CO₂, 5 % O₂ and 90 % N₂ of humidified air.

Nuclear chromatin evaluation

At the end of maturation, cumulus cells were removed from oocytes using hyaluronidase (600 IU/ml) in TCM-199 and subsequently oocytes were stained with 2.5 mg mL⁻¹ Hoechst 33258 in 3:1 (v/v) glycerol/PBS. The presence of the second metaphase plate and first polar body or two independent areas of fluorescing chromatin in the oocytes were classified as metaphase-II.

RNA extraction, cDNA synthesis and real time PCR

Total cellular RNA was extracted from the denuded mature oocytes (pooled of $n=20$ oocytes for each group) using TRI-reagent. Synthesis of cDNA was carried out with M-MuLV reverse transcriptase (RT) and random hexamer as primer, according to the manufacturer's instructions (Fermentas; St. Leon-Rot, Germany). Real-Time quantitative reverse transcription PCR (RT-PCR) reactions of 25 µl were conducted in Rotor Gene 6000 (Corbett Life Science, Sydney, Australia), by adding 12.5 µl 2X SYBR Premix Ex Taq, 0.4 µM of final concentration for each primer, 2 µl template, and distilled water to reach the volume of 25 µl. Real-time PCR was performed in two steps with the following thermal setting: 3 min at 95 °C for initial enzyme activation followed by 40 amplification cycles (each 5 s at 95 °C, and 20 s at 60 °C with fluorescence detection), and a final step of melting curve analysis. The information for primers used for real-time PCR is listed in Table 1. All the samples were analyzed in duplicate, and the average value of the duplicate was used for quantification. The data were

normalized to *GAPDH* and 2- $\Delta\Delta C_t$ methodology was used for relative quantification [17].

Oocyte activation

After IVM, a number of COCs were denuded and washed in TCM-199 supplemented with 10 % FBS and activated chemically by applying 1 min exposure to 2.5 µM ionomycin (diluted in TCM-199) followed by 2 mM 6-DMAP (diluted in CR1aa medium) for 3 h [18].

In vitro culture

Following activation in vitro, denuded presumptive zygotes were washed three times in CR1aa medium. Groups of 15 to 20 zygotes were cultured in a 30 µl CR1aa (2 % BME-essential amino acids, 1 % MEM-nonessential amino acids) medium drops supplemented with 10 % FBS for 8 days at 38.5 °C in a humidified incubator with 5 % O₂, 5 % CO₂, and 90 % N₂. The day of activation was considered as day 0.

Experimental design

Experiment 1. Evaluation of ALA concentration in small and large follicles

In order to find an appropriate range of ALA for supplying in prospective experiments, 1 ml of FF samples were aspirated from small (≤ 2 mm) and large (≥ 6 mm) follicles and the fatty acid composition of the FF was evaluated. GC-MS was used to determine the composition of fatty acids and compare the differences of fatty acids between small- and large-follicle groups. Six replicates, each containing 1 ml of FF, were analyzed for this experiment.

Experiment 2. Effects of exogenous ALA in maturation media on meiotic maturation after IVM

Based on ALA concentrations found in Experiment 1, the concentrations of 10, 50, 100, and 200 µM ALA have been chosen for use in this experiment. Therefore, COCs were randomly assigned to each of ALA-10 ($n=111$), ALA-50 ($n=$

Table 1 Details of primers used for real time PCR quantitative analysis

Gene name	Primer Sequences	Annealing temperature (°C)	Product size (bp)
<i>Bax</i>	F:5'-TGGAGATGAATTGGACAGTAAC-3' R:5'-GCCTTGAGCACCAGTTTG-3'	61	174
<i>Bcl-2</i>	F:5'-ATGTGTTGGAGAGCGTCA-3' R:5'-AGAGACAGCCAGGAGAAATC-3'	60	182
<i>GAPDH</i>	F:5'-GGAGGGACTTATGACCACTG-3' R:5'-CTGCTTCACCACCTTCTTG-3'	60	135

F forward, PCR polymerase chain reaction, R reverse

117), ALA-100 ($n=98$), and ALA-200 ($n=103$) groups. In addition, a number of COCs ($n=121$) not supplemented with ALA were considered as control group. After IVM, COCs from each group were evaluated for the stage of nuclear maturation. Three replicates were performed per treatment.

Experiment 3. Effects of exogenous ALA in maturation media on mRNA abundance of Bax, and Bcl-2 genes in mature oocytes

To evaluate the effect of ALA on cell apoptosis induction, mRNA abundance of apoptosis-related genes was analyzed in mature oocyte. After IVM, oocytes at Metaphase-II stage derived from different concentrations of ALA as well as oocytes from control group were used for the evaluation of pro-apoptotic (*Bax*) and anti-apoptotic (*Bcl-2*) mRNA abundance. Three replicates were analyzed for this experiment.

Experiment 4. Effects of exogenous ALA in maturation media on oocyte developmental potential

To determine whether ALA has positive effects on embryonic developmental potential, matured oocytes were activated, and cultured in vitro. Cleavage rate (i.e., progression to the two-cell stage) and blastocyst development were recorded and compared between the ALA-treated groups and the control group at day 3 and 8 post-culture, respectively. The day of activation was considered as day 0. This experiment was executed in three replicates.

Statistical analyses

Differences among the mean values were tested using ANOVA followed by Duncan's multiple range test. The relative expression data were analysed using General Linear Model (GLM) of the Statistical Analysis System (SAS) software package version 8.0 (SAS Institute Inc., NC, USA). Differences of $P>0.05$ were considered to be significant.

Results

Experiment 1. Evaluation of ALA concentration in small and large follicles

Our finding revealed that the concentration of ALA significantly increased as the ovarian follicles enlarged. Comparative analysis showed that concentration of ALA in follicular fluids was lower ($P<0.05$) in small (≤ 2 mm) follicles (2.1 ± 0.3 mg/100 ml; 75.4 μ M) as compared with large (≥ 6 mm) follicles (3.5 ± 0.5 mg/100 ml; 125.7 μ M).

Experiment 2. Effect of exogenous ALA in maturation medium on meiotic maturation after IVM

Supplementation of maturation medium with 200 μ M ALA resulted in the lowest ($P<0.05$) maturation rate as compared with the control group (71.3 ± 3.5 vs. 83.4 ± 3.2). However, maturation rates were similar ($P>0.05$) among ALA-10 (83.6 ± 1.4), ALA-50 (86.8 ± 1.7), ALA-100 (89.1 ± 4.1), and control groups.

Experiment 3. Effects of exogenous ALA in maturation medium on expression of Bax, and Bcl-2 genes in mature oocytes

Transcript data for pro-apoptotic (*Bax*) and anti-apoptotic (*Bcl-2*) genes in mature oocytes are shown in Figs. 1 and 2, respectively. The mRNA abundance of *Bax* in mature oocytes was decreased ($P<0.05$) in ALA-50, ALA-100, and ALA-200 groups when compared with ALA-10 and control groups (Fig. 1). Furthermore, there was an increased ($P<0.05$) in mRNA abundance of *Bcl-2* transcripts in mature oocytes in ALA-100, and ALA-200 groups when compared with the ALA-10, ALA-50, and control groups (Fig. 2).

Experiment 4. Effects of exogenous ALA in maturation media on oocyte developmental potential

Proportional data for cleavage and development to the blastocyst following parthenogenetic activation are shown in Table 2. Although the cleavage rates were similar ($P>0.05$) among ALA-treated and control groups, oocytes in ALA-100 group produced more ($P<0.05$) blastocysts than ALA-10, ALA-50, ALA-200 and control group.

Fig. 1 Relative mRNA expression of *Bax* transcripts of ALA-treated oocytes compared with control oocytes after IVM. Data are given as the mean $\pm$ SD. ^a, ^bBars with different letters represent groups that were different ($P<0.05$)

Fig. 2 Relative mRNA expression of *Bcl-2* transcripts of ALA-treated oocytes compared with control oocytes after IVM. Data are given as the mean $\pm$ SD. ^{a, b}Bars with different letters represent groups that were different ($P<0.05$)

Discussion

To investigate the variation of ALA concentration in ovarian follicles and its relationship to the follicular size, antral follicles were categorized into “small” and “large” groups and fatty acid composition of each group was estimated by gas chromatography. This study found that significant differences do exist between small and large follicles with respect to ALA. The amount of ALA increased as the ovarian follicles enlarged. This finding might be expected by assuming a greater need for some specific unsaturated fatty acids as follicles grow and mature.

To evaluate the effect of ALA on oocyte nuclear maturation, exogenous ALA was added to the maturation medium, in a dose dependent manner. Concentrations of ALA in follicular fluid were 0.021 mg/ml (75.4 μ M) and 0.035 mg/ml (125.7 μ M) for small and large follicles, respectively. Therefore, a range of 0, 10, 50, 100, and 200 μ M ALA was supplemented in IVM medium to accurately mimic the normal

physiological situation in vitro. The addition of ALA had no effect on meiotic maturation except at the highest concentration (200 μ M), in which a detrimental effect was detected. Supporting our current findings, a previous study [2] demonstrated that supplementation of maturation medium with ALA at a concentration lower than 100 μ M had no adverse effect on oocyte meiotic maturation. The deleterious effect of ALA at a concentration of 200 μ M in the present study is consistent with previous reports in which a concentration higher than 100 μ M decreased MII rate after 24 h IVM [15]. The lowest maturation rate at highest concentration of ALA (200 μ M) might be explained by the toxicity effect of fatty acids outside the tolerance threshold of the cells, leading to a reduction in cell viability [19].

Bcl-2 family proteins are involved in the regulation of apoptosis and proposed to integrate signals from survival-inducing and death-promoting pathways [20, 21]. To elucidate the effect of ALA on oocyte apoptosis, we analyzed the mRNA abundance of *Bax*, and *Bcl-2* in oocyte after IVM. The analysis of transcript abundance of candidate apoptosis-related genes revealed variability between ALA-treated and control oocytes. Our results demonstrated that ALA-treated oocytes showed a decrease in *Bax* mRNA level and an increase in abundance of the *Bcl-2* mRNA compared to oocytes of the control group. *Bax* is a pro-apoptotic *Bcl-2* family member whose apoptotic function is antagonized by *Bcl-2* abundance [22]. Our data revealed a decrease abundance of pro-apoptotic (*Bax*) and increase abundance of anti-apoptotic (*Bcl-2*) transcripts in ALA-treated oocytes compared to the control oocytes. An earlier study in cattle [23] revealed a decreased level of reactive oxygen species following supplementation of the maturation medium with ALA which may also explain the higher apoptosis-suppressing effect of ALA observed in this study.

Although parthenogenetic activation of oocytes does not necessarily evoke the complete physiological process, it is a useful tool for understanding particular early embryonic developmental processes [24]. Therefore in this study a parthenogenetic model of preimplantation development was used to evaluate the effect of ALA on oocyte developmental competence in ovine. After parthenogenetic activation, the cleavage rate was not affected within the treatment groups; however blastocyst rate was higher for 100 μ M ALA treated oocytes in comparison with ALA-10, ALA-50, ALA-200 and controls oocytes. In contrast to our findings, in pre-pubertal sheep, no differences were observed in blastocyst development among control, 50, 100, and 200 μ M ALA-treated oocytes, but an improvement in male pronuclear formation in zygotes was observed [15]. However, similar to the findings of our study, an increased rate of embryonic development has been observed for bovine oocytes, although an optimum range of ALA in that study showed to be 50 μ M [3]. In another study in cattle, addition of ALA at 1, 10, and 100 μ M concentrations

Table 2 Effect of ALA on embryonic developmental potential of ovine oocytes after parthenogenetic activation (cumulative results of three replicates)

Groups	No. Oocytes	Embryo development	
		Cleaved n (%)	Blastocyst n (%)
Control	114	85 (74.4)	33 (28.8) ^c
ALA-10	110	82 (74.5)	34 (31.0) ^{bc}
ALA-50	118	91 (77.2)	41 (34.7) ^b
ALA-100	123	96 (78.0)	48 (39.0) ^a
ALA-200	106	77 (72.6)	23 (21.7) ^d

ALA-10 10 μ M α -linolenic acid, ALA-50 50 μ M α -linolenic acid, ALA-100 100 μ M α -linolenic acid, ALA-200 200 μ M α -linolenic acid

^{a,b,c,d} Different superscripts within the columns indicate a significant difference ($P<0.05$)

to the culture medium did not improve the cleavage and blastocyst rates [25]. These different findings suggest that ALA might be more significant in the time of maturation. Furthermore, Al Darwich et al. (2010) also found that the viability of the produced bovine embryos was influenced in a positive manner by the presence of ALA in culture medium [25] that may be reflect the anti-apoptotic effect of ALA on mRNA abundance of the *BAX* and *BCL-2*.

It is now becoming increasingly apparent that, apart from producing energy for oocytes and early embryos, fatty acids are an important component of some biochemical pathways. This includes re-initiation and completion of the first meiotic division and cytoplasmic maturation, which is critical for normal fertilization and further embryonic development [26]. In this regard, supplementation of maturation medium with ALA increased the synthesis of PGE2 by enhancing the total concentration of ω -6 fatty acids as precursors [3]. Furthermore, ALA has been reported to increase both PGE2 and PGF2 α concentrations in the culture medium of pre-pubertal sheep oocytes [15]. PGE2 is a major paracrine and autocrine regulator of cumulus cell functions and has been proposed to play an important function in oocyte nuclear maturation [27]. As observed in a previous study, an elevated concentration of PGE2 positively affected cumulus expansion and nuclear maturation by enhancing the phosphorylation of MAPK1 and MAPK2 in both oocytes and cumulus cells through the elevation of cAMP concentrations [3].

In conclusion, our data demonstrated that supplementation of maturation medium with exogenous 100 μ M ALA increased in vitro blastocyst formation. ALA-treated medium led to an improvement in oocyte competence, thus, shall lead to further investigations regarding the ALA mechanisms during in vitro oocyte/embryo culture which could be in the near future applied in routine ART's programs.

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