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Original Article

Effects of Essential Fatty Acids during *In Vitro* Maturation of Porcine Oocytes: Hormone Synthesis and Embryonic Developmental Potential

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ABSTRACT   Omega-3 α-linolenic acid and omega-6 linoleic acid are essential fatty acids for health maintenance of human and animals because they are not synthesized *in vivo*. The purpose of this study was to evaluate the effect of α-linolenic acid and linoleic acid supplementation on *in vitro* maturation and developmental potential of porcine oocytes. Various concentrations of α-linolenic acid and linoleic acid were added into *in vitro* maturation medium, and we evaluated the degree of cumulus expansion, oocyte nuclear-maturation rate, blastocyst rate, blastocyst quality, and levels of prostaglandin E_2, 17β-estradiol, and progesterone in the spent medium. High doses (100 μM) of α-linolenic acid and linoleic acid supplementation significantly inhibited cumulus expansion and oocyte nuclear maturation, and prostaglandin E_2 synthesis also significantly decreased compared with other groups ($p < 0.05$). Supplementation of 50 μM α-linolenic acid and 10 μM linoleic acid showed higher quality blastocysts in terms of high cell numbers and low apoptosis when compared with other groups ($p < 0.05$), and synthesis ratio of 17β-estradiol / progesterone also significantly increased compared with control group (3.59 ± 0.22 vs. 2.97 ± 0.22, 3.4 ± 0.28 vs. 2.81 ± 0.19, respectively; $p < 0.05$). Our results indicated that supplementation with appropriate levels of α-linolenic acid and linoleic acid beneficially affects the change of hormone synthesis (in particular, an appropriate increase in the 17β-estradiol / progesterone synthesis ratio) for controlling oocyte maturation, leading to improved embryo quality. However, high doses of α-linolenic acid and linoleic acid treatment results in detrimental effects.

Keywords: α-linolenic acid, linoleic acid, porcine oocytes, prostaglandin, 17β-estradiol / progesterone

INTRODUCTION

Oocytes maturation is critical factor in determining its developmental potential and embryo quality produced after fertilization, and appropriate steroid and eicosanoid secretion are essential for optimal maturation (Sirard et al., 2006). During folliculogenesis until the LH surge, the concentration of 17β-estradiol (E2) is high in the follicular fluid. Subsequently, the LH surge stimulates the transformation of theca and granulosa cells into luteal cells, which begin to produce progesterone (P4) instead of E2 (Walsh et al., 2012). Therefore, sex steroids (such

as E2 and P4) present in follicular fluid might be key factors that determine the fate of the oocytes (Hyttel et al., 1986). It has been well demonstrated that E2 is essential during follicular growth (Lubahn et al., 1993). However, high E2 concentrations during final maturation induce oocyte nuclear aberrations and inhibit meiotic progression in bovine species (Beker-van et al., 2004). It has been also demonstrated that P4 is an important factor in oocyte development and ovulation (Lydon et al., 1995). Additionally, supplementation of P4 into maturation medium stimulates meiotic resumption in bovine (Siqueira et al., 2012) and porcine (Yamashita Y et al., 2003) oocytes. However, Wathes et al. (2007) also reported that increased P4 in IVM medium has a negative effect on oocyte-developmental potential, resulting in the production of poor-quality embryos. Modina et al. (2014) reported that the E2/P4-synthesis ratio in follicular fluid has been used as a surrogate marker for healthy and atretic follicles. These results suggested that E2 and P4 seem to act in opposition to nuclear maturation, and furthermore, balance in optimal hormone levels in follicular fluid during final maturation is crucial for oocyte development and the production of good-quality embryos. One of the eicosanoids, prostaglandins (PGs) playing important roles in reproductive functions, such as estrous, ovulation, embryo survival, and parturition (Abayasekara et al., 2009), and serving as critical mediators during oocyte maturation (Neal et al., 1975). Prostaglandin E_2 (PGE_2) is the dominant PG in the preovulatory follicles (Segi et al., 2003), and many studies have shown that PGE_2 plays important roles in cumulus-cell expansion and oocyte maturation in rodents (Takahashi et al., 2006) and cattle (Nuttinck et al., 2011). Hizaki et al. (1999) reported abortive expansion of the cumulus and impaired fertility in mice lacking the PGE-receptor. Additionally, PGE_2 is thought to act as luteotrophic factor in the early luteal phase (Ghaffarilaleh et al., 2014), and are known to affect steroid hormone secretion indirectly (Wathes et al., 2007).

It has been shown that changes in the type and concentrations of polyunsaturated fatty acids (PUFAs) in diet can alter ovarian environment through such as changes in steroid and eicosanoid secretion, and such alternation also affects the oocyte maturation and embryo developmental potential and furthermore the pregnancy rate (Mattos et al., 2000). Although PUFAs have important effects on reproductive function because they may be involved in reproductive process as precursor of steroid hormone (via cholesterol) and of PGs (via arachidonic acid), very few studies have been provided detailed descriptions of their nature and mechanism. Importantly, different types of PUFA supplementation have differential effects on reproductive function (Gulliver et al., 2012). PUFAs have more than one double bond present within the molecule, and omega-3 and omega-6 series are classified according to the location of the first double bond (Wathes et al., 2007). The three types of omega-3 fatty acids involved in human physiology are a-linolenic acid (ALA: C18:3), eicosapentaenoic acid (EPA: C20:5), and docosahexaenoic acid (DHA: C22:6). The four types of omega-6 fatty acids involved in human physiology are linoleic acid (LA: C18:2), gamma-linolenic acid (GLA: C18:3), dihomo-gamma-linolenic acid (DGLA: C20:3), and arachidonic acid (AA: C20:4). In mammals, ALA can be transformed to EPA and DHA, LA can be transformed to DGLA and AA. However, animals cannot synthesize ALA and LA *de novo*, as they lack the appropriate fatty acid desaturase enzymes. Therefore, both need to be provided in the diet, given their requirement for numerous processes, including growth, reproduction, vision, and brain development (Wathes et al., 2007). For this reason, they are called essential fatty acids (EFAs) (Wiktorowska-Owczarek et al., 2015).

Most omega-3 PUFAs are derived in ALA, localizes in the plasma and follicular fluid (Wathes et al., 2007) and positively affects oocyte development (Childs et al., 2008). Veshkini et al. (2015) found that the amount of ALA increases as ovarian follicles enlarge. Additionally, ALA is involved in the regulation of meiotic arrest at the germinal vesicle (GV) stage development (Wathes et al., 2007). Bovine oocyte supplementation with ALA during IVM results in an increased maturation rate, a higher blastocyst yield, and production of better-quality blastocyst embryos (Marei et al., 2009). Furthermore, during IVM, COCs treated with ALA show significant increases in PGE_2 concentration in the spent medium. Similarly, Ghaffarilaleh et al. (2014) reported that ALA affects prepubertal sheep-embryo quality associated with alterations in released reproductive hormones. Another EFA, omega-6 LA is abundant in follicular fluid and detrimentally affects oocyte development. LA-supplemented feed negatively affects fertility in cattle as compared with ALA supplementation (Santos et al., 2008), and Marei et al. (2010) reported that treat-

ment of COCs with LA during IVM significantly inhibits cumulus-cell expansion and retards oocyte development at the metaphase II (MII) stage in a dose-dependent manner. LA also significantly lowers the percentage yield of cleaved embryos and blastocyst embryos, and increases PGE_2 concentration in the spent medium. LA-induced alterations in mitochondrial distribution and activity, as well as increases in levels of reactive oxygen species, partially mediate the inhibitory effect on oocyte maturation (Marei et al., 2012). By contrast, positive reports associated with addition of appropriate concentrations of LA to a defined maturation medium showed no alterations in the nuclear status of bovine oocytes matured *in vitro*, but improved their quality by increasing the neutral lipid content stored in lipid droplets (Carro et al., 2014). Moreover, fatty acid-content analysis of bovine follicular fluid collected from different-sized follicles showed significantly lower LA concentration in large follicles as compared with small follicles (Homa et al., 1992). These reports revealed that the physiological decrease in LA concentration in follicular fluid might be important to oocyte maturation, and that LA might play a role in controlling oocyte maturation. Consequently, the types and quantity of PUFAs added during IVM might play an important mediatory role in oocyte maturation by inducing changes in PGs, E2, and P4 synthesis, which could result in the production of high-quality blastocyst embryos.

In order to investigate the effect of EFA on oocyte maturation, we assessed whether supplementation with the ALA and LA to porcine oocyte IVM medium affected cumulus expansion and oocyte nuclear-maturation rate, developmental rate to the blastocyst stage, and blastocyst quality, as well as concentrations of PGE_2 and sex steroids (E2 and P4) in the spent medium.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). Medium for oocyte washing, maturation, and culture was pre-incubated at 38.5°C and 5% CO_2 in an air incubator for at least 4 h before use.

Oocyte collection and *in vitro* maturation

Porcine ovaries were collected from a local abattoir and transported to the laboratory in a thermos container in 0.9% saline supplemented with 75 μg/mL potassium penicillin G at between ~25°C and 30°C within 2 h after killing. They were washed in fresh 0.9% saline supplemented with 75 μg/mL potassium penicillin G immediately after arrival. COCs were aspirated through an 18-gauge needle into a disposable 10-mL syringe from follicles 3 mm to 6 mm in diameter (Homa et al., 1994). After washing three times with HEPES-buffered Tyrode's medium (TL-HEPES), ~50 COCs were matured in 500 μL IVM medium in a 4-well multi-dish (Nunc, Roskilde, Denmark) at 38.5°C and 5% CO_2 in an air incubator. The basic medium used for oocyte maturation was North Carolina State University medium 23 (NCSU-23) supplemented with 6 mg/mL fatty acid-free bovine serum albumin (BSA; Sigma-Aldrich) , 0.57 mM cysteine, 10 ng/mL β-mercaptoethanol, 10 ng/mL epidermal growth factor, 10 IU/mL pregnant mare's serum gonadotropin (PMSG), and 10 IU/mL human chorionic gonadotropin (hCG) (Petters et al., 1993). After 22 h of culture, oocytes were washed three times and then further cultured in maturation medium in the absence of hormone supplements (PMSG or hCG) for 22 h. After completion of IVM, oocytes were subjected to *in vitro* fertilization.

In vitro fertilization and culture

In vitro fertilization of porcine oocytes was performed as described previously (Abeydeera et al., 1997). Fresh semen was kindly supplied once a week by the AI Company (Darby Pig AI Center, Anseong, Korea) and stored at 17°C for 5 days. Semen was washed three times by centrifugation with Dulbecco's phosphate-buffered saline (DPBS; Gibco BRL, Grand Island, NY, USA) supplemented with 1 mg/mL BSA (Fraction V; Sigma-Aldrich), 100 mg/mL penicillin G, and 75 mg/mL streptomycin sulfate. After washing, spermatozoa were resuspended in modified Tris-buffered medium (mTBM) at pH 7.8. Oocytes were washed three times in mTBM with 2.5 mM caffeine sodium benzoate and 1 mg/mL BSA (fatty acid free) and placed into 48 μL mTBM under paraffin oil, followed by addition of 2 μL of diluted spermatozoa to the 48-μL drop of the medium containing between ~15 and ~20 oocytes at a final concentration of 1.5×10^5 sperm/mL. The oocytes were co-incubated with spermatozoa for 6 h at 38.5°C and 5% CO_2 in an air incubator. After fertilization, the embryos were cultured in 50-μL drops of porcine zygote medium-3

(PZM-3) supplemented with 3 mg/mL BSA at 38.5°C and 5% CO_2 in an air incubator. After 48 h of culture, 25 to 30 cleaved embryos were further cultured in 50 μL PZM-3 medium supplemented with 3 mg/mL BSA for 4 days. Blastocyst embryos were observed after 6 days of culture.

Oocyte-maturation assessment: cumulus-cell expansion and nuclear stage

After 44 h of maturation, the degree of cumulus expansion was assessed subjectively using a stereomicroscope and qualified as not expanded, partially expanded (the outer layer of cells was loosened), or fully expanded (all cumulus cells were loosened). Additionally, to assess the stage of nuclear maturation at the end of the maturation time, oocytes were stained with aceto-orcein as described previously (Marei et al., 2009). Representative samples of oocytes were denuded by gentle pipetting in NCSU-23 medium containing 0.1% hyaluronidase, washed in DPBS supplemented with 0.1% BSA, and mounted on microscope slides. The samples were fixed in acetic acid:ethanol (1:3, v/v) after 3 days and stained with acetic orcein 0.1% (v/v) for 5 min. The samples were destained in glycerol:acetic acid:water (1:1:3, v/v/v), and the nuclear stage was evaluated under a microscope at 200× and 400 × magnification (Leica, Solms, Germany).

PGE$_2$ radioimmunoassay

Conditioned maturation medium was collected after 44 h of culture and stored at -20°C. PGE_2 concentration was quantified using charcoal-dextran-coated radioimmuno-assay methods as described previously (Marei et al., 2009). Briefly, standards (range: ~0.05-10 ng/mL) or samples were mixed with anti-PGE_2 serum (from Dr. N.L. Poyser, University of Edinburgh, Edinburgh, United Kingdom) and tritiated tracer ([5, 6, 8, 11, 12, 14, 15 (n)-3H]-PGE_2; Amersham International, Amersham, United Kingdom) in duplicate. After overnight incubation at 4°C, dextran-coated charcoal suspension containing 0.4% dextran (T-70; Amersham Pharmacia Biotech, Uppsala, Sweden) and 2% neutralized charcoal was added to all tubes, except the that used for the total count. Tubes were incubated at 4°C for 10 min, followed by centrifugation at 2000 × g for 10 min. The supernatant was removed into 6-mL scintillation vials containing 4 mL scintillation fluid (Ultima Gold; Packard Bioscience BV, Pangbourne, United Kingdom) and counted for 2 min. PGE_2 concentration was calculated using a semi-logarithmic plot at a limit of detection of 2 pg/tube. The intra-assay coefficient of variation was 3.5%, whereas the inter-assay coefficient was 6.3%.

Measurement of E2 and P4 by enzyme-linked immunosorbent assay (ELISA)

The steroidogenic activity of cumulus cells was assessed in the maturation medium according to the procedure previously described by Maya-Soriano et al. (2013) Briefly, E2 and P4 concentrations were determined after 44 h of IVM using commercial enzyme immunoassay kits (estra-diol ELISA kit 402110 and progesterone (ultra) ELISA kit 402310, respectively; Neogen Corporation, Lexington, KY, USA) according to manufacturer instructions. The assay was validated by determination of assay specificity (dilu-tional 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).

Assessment of embryo quality

Apoptotic cells in blastocyst embryos were detected us-ing an *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany). Blastocyst embryos were washed three times in DPBS with 0.1% polyvinylpyrrolidone and fixed in 4% (v/v) paraformaldehyde diluted in DPBS for 1 h at between ~24°C and ~28°C. For membrane permea-bilization, fixed embryos were incubated in DPBS con-taining 0.1% (v/v) Triton X-100 for 30 min at 4°C. Fixed embryos were incubated in terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (TUNEL) reaction medium for 1 h at 38.5°C in the dark, washed, transferred into 2 mg/mL 4′,6-diamidino-2-phenylindole (DAPI), and mounted onto slides. Whole-mount embryos were examined using an epifluorescence microscope (Olympus, Tokyo, Japan) to assess the TUNEL assay and DAPI staining. The number of apoptotic nuclei and the total number of nuclei were counted.

Statistical analysis

Binominal data from oocyte nuclear maturation, cleav-age rate, and developmental rate of blastocyst embryos were converted into percentages. These data were then analyzed using the Student's *t* test. Data for the cumu-lus expansion, total and apoptotic cell numbers, PGE_2
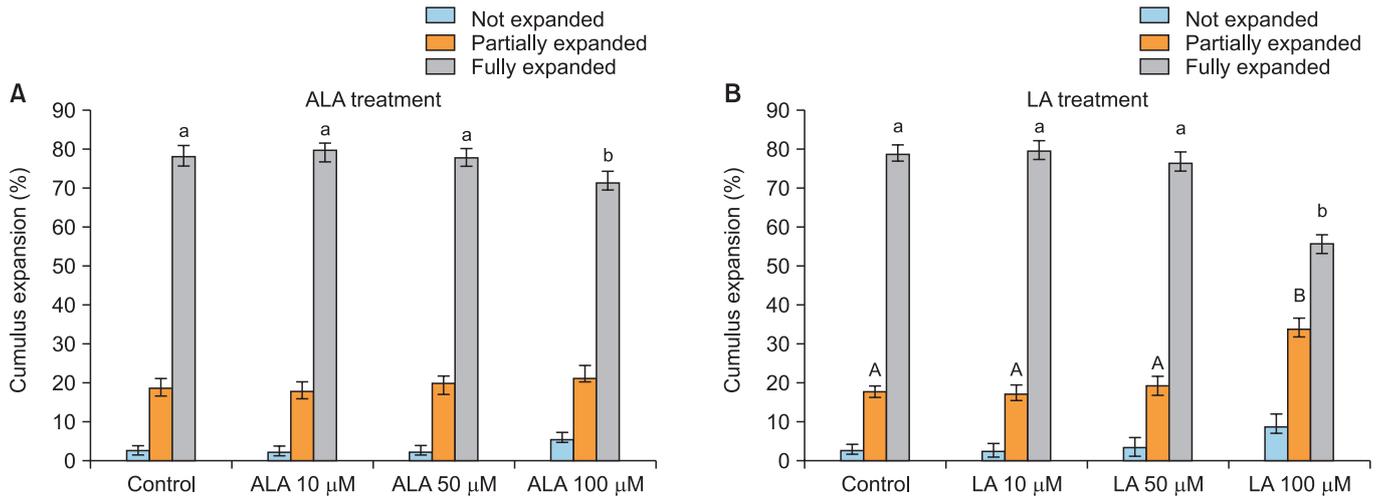
Fig. 1. Effect of ALA and LA concentrations on cumulus-cell expansion during IVM of porcine follicular oocytes. Cumulus-cell expansion of COCs treated in the presence or absence of (A) ALA or (B) LA for 44 h. Results are presented as the mean ± SEM of seven replicates. Different superscript letters indicate significant differences between experimental groups ($p < 0.05$). ALA; a-linolenic acid, COC; cumulus-oocyte complex, IVM; *in vitro* maturation, LA; linoleic acid, SEM; standard error of the mean.

Table 1. Effect of ALA concentrations during IVM on nuclear maturation of porcine follicular oocytes *in vitro*

Concentration of ALA	No. of oocytes	No. (%) of developed to MII stage oocytes
Control	360	282 (78.3)[a]
10 μM	382	304 (79.6)[a]
50 μM	374	291 (77.8)[a]
100 μM	370	254 (68.7)[b]

[a,b]Values with different superscripts are significantly different ($p < 0.05$).
ALA; a-linolenic acid, IVM; *in vitro* maturation.

Table 2. Effect of LA concentrations during IVM on nuclear maturation of porcine follicular oocytes *in vitro*

Concentration of LA	No. of oocytes	No. (%) of developed to MII stage oocytes
Control	341	269 (79.1)[a]
10 μM	375	300 (80.0)[a]
50 μM	360	277 (77.1)[a]
100 μM	370	208 (56.2)[b]

[a,b]Values with different superscripts are significantly different ($p < 0.05$).
LA; linoleic acid, IVM; *in vitro* maturation.

amount, and E2/P4-synthesis ratio were analyzed using a general linear model analysis of variance and reported as the mean ± standard error of the mean (SEM). A $p < 0.05$ was considered statistically significant.

RESULTS

Cumulus expansion and oocyte nuclear maturation

The effect of ALA or LA supplementation to IVM medium on cumulus expansion, and oocyte nuclear maturation is shown in Fig. 1, Tables 1 and 2. The highest concentration (100 μM) of ALA and LA significantly decreased the expansion rate of fully expanded cumulus cells at 44 h after IVM as compared with each of the other groups, with the reduction in rate slightly higher in the LA-treatment group ($p < 0.05$; Fig. 1). The results from oocyte maturation did not show differences in the percentage of oocytes

at the MII stage at 44 h of IVM between the 0 μM, 10 μM, and 50 μM ALA- and LA-treatment groups. By contrast, significantly lower rates of MII-stage oocytes were observed in the 100 μM ALA- and LA-treatment groups as compared with the other groups ($p < 0.05$; Tables 1 and 2).

PGE₂ synthesis

Fig. 2 shows the PGE_2 concentration in the spent medium after IVM of 100 porcine follicular oocytes for 44 h treated with 0 μM, 10 μM, 50 μM, or 100 μM ALA or LA. The 10 μM, 50 μM, and 100 μM ALA-treatment groups showed significantly decreased PGE_2 concentrations as compared with the control group and in a dose-dependent manner ($p < 0.05$), whereas the 10 μM and 50 μM LA-treatment groups showed significantly increased PGE_2 concentrations as compared with the control group and in a dose-dependent manner ($p < 0.05$). However, the 100
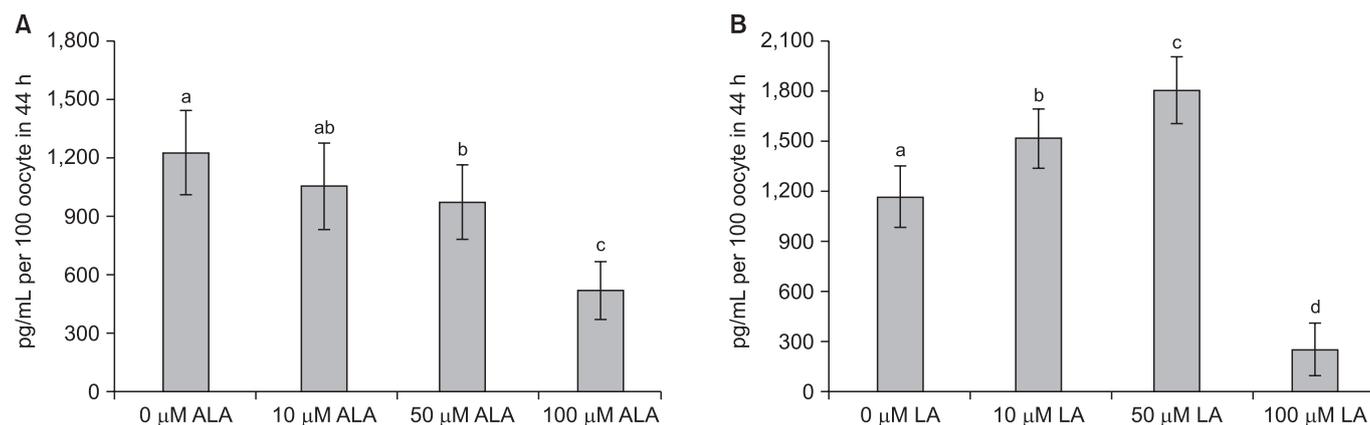
A

B

Fig. 2. Effect of ALA and LA concentrations on PGE_2 synthesis during IVM of porcine follicular oocytes. PGE_2 synthesis of COCs treated in the presence or absence of (A) ALA or (B) LA for 44 h. Results are presented as the mean ± SEM of seven replicates. Different superscript letters indicate significant differences between experimental groups ($p < 0.05$). ALA; a-linolenic acid, IVM; *in vitro* maturation, LA; linoleic acid, PGE2; prostaglandin E_2, SEM; standard error of the mean.

Table 3. Effect of ALA concentrations during IVM on embryonic development of porcine follicular oocytes *in vitro*

Concentration of ALA	No. of oocytes	No. (%) of cleaved oocytes	No. (%) of blastocyst embryos developed from cleaved oocytes
Control	968	808 (83.5)[a]	256 (31.7)[a]
10 µM	1,000	848 (84.8)[a]	279 (32.9)[a]
50 µM	968	816 (84.3)[a]	264 (32.4)[a]
100 µM	984	784 (79.7)[b]	230 (29.3)[b]

[a,b]Values with different superscripts are significantly different ($p < 0.05$).
ALA; a-linolenic acid, IVM; *in vitro* maturation.

Table 4. Effect of LA concentrations during IVM on embryonic development of porcine follicular oocytes *in vitro*

Concentration of LA	No. of oocytes	No. (%) of cleaved oocytes	No. (%) of blastocyst embryos developed from cleaved oocytes
Control	741	606 (81.7)[a]	182 (30.0)[a]
10 µM	705	559 (79.3)[a]	171 (30.6)[a]
50 µM	729	571 (78.3)[a]	160 (28.0)[a]
100 µM	690	451 (65.4)[b]	67 (14.9)[b]

[a,b]Values with different superscripts are significantly different ($p < 0.05$).
LA; linoleic acid, IVM; *in vitro* maturation.

µM LA-treatment groups showed significantly decreased PGE_2 concentrations as compared with the control group ($p < 0.05$).

Cleavage and embryo development

The effect of ALA and LA concentrations during IVM on embryonic development of porcine follicular oocytes *in vitro* is shown in Tables 3 and 4. The percentage of oocytes that cleaved into 4-cell stage embryos at 48 h after fertilization and the percentage of cleaved oocytes that developed to the blastocyst stage at day 6 after fertilization were recorded. No differences were found in the cleavage rate or blastulation rate between the control and 10 µM and 50 µM ALA- and LA-treatment groups, however, the 100 µM ALA- and LA-treatment group showed significantly lower cleavage and blastulation rates as compared with the other groups ($p < 0.05$; Table 3 and 4).

Embryo quality

The effect of ALA and LA concentrations during IVM on the quality of *in vitro*-produced porcine blastocyst embryos assessed by the number of total and apoptotic cells is shown in Tables 5 and 6. The number of total and apoptotic cells from *in vitro*-produced porcine blastocyst embryos at 6 days after fertilization was recorded. Both the 50 µM ALA- and 10 µM LA-treatment groups showed significantly higher numbers of total cells and lower numbers of apoptotic cells as compared with the other groups ($p < 0.05$; Tables 5 and 6). Apoptosis was measured using the TUNEL assay, which allows for the identification of fragmented DNA. As shown in Fig. 3, apoptotic nuclei appeared green.

E2/P4-synthesis ratio

Fig. 4 shows the E2/P4-synthesis ratio in the spent medium after IVM from 100 porcine follicular oocytes in

the presence of 0 μM, 10 μM, 50 μM, or 100 μM ALA and LA. Both the 50 μM and 100 μM ALA-treatment groups showed significantly higher E2/P4-synthesis ratios as compared with the control group and in a dose-dependent manner ($p < 0.05$; Fig. 4). Moreover, the 10 μM LA-treatment group showed a significantly higher E2/P4-synthesis ratio as compared with the other groups ($p < 0.05$), whereas the 100 μM LA-treatment group showed a significantly lower E2/P4-synthesis ratio was compared with the other groups ($p < 0.05$).

DISCUSSION

To explain the effect of ALA and LA on nuclear, cytoplasmic, and physiological changes occurring during oocyte maturation, we measured alterations in oocyte maturation rate, cumulus-cell expansion, and embryo-

developmental potential after fertilization. Additionally, we investigated changes in PGE$_2$ and sex-steroid (E2 and P4) synthesis in the spent media.

Our study found that supplementation with 10 μM and 50 μM ALA did not affect oocyte nuclear maturation or cumulus expansion, whereas 100 μM ALA supplementation resulted in a significant inhibitory effect (Table 1 and Fig. 1A). These results implied that the highest experimental dose of ALA supplementation inhibited IVM of porcine COCs. A similar effect was observed for 200 μM ALA supplementation in the IVM medium of prepubertal sheep oocytes, where there was a negative effect on nuclear maturation and cumulus cell expansion (Ghaffarilaleh et al., 2014). In a bovine study conducted by Marei et al. (2009), COC treatment with 50 μM ALA significantly increased oocyte percentages at the MII stage as compared with that observed in the untreated control group. However, high doses (100 μM and 200 μM) of ALA treatment

Table 5. Effect of ALA concentrations during IVM on the number of total cells and apoptotic cells produced by porcine blastocyst embryos *in vitro*

Concentration of ALA	No. of examined blastocyst embryos	No. of total cells in blastocyst embryos	No. of apoptotic cells in blastocyst embryos
Control	25	41.8 ± 3.1[a]	1.5 ± 0.9[a]
10 μM	28	41.4 ± 2.9[a]	1.3 ± 1.1[a]
50 μM	29	47.4 ± 2.7[b]	0.9 ± 0.8[b]
100 μM	23	40.1 ± 3.2[a]	1.5 ± 1.2[a]

Results are presented as the mean ± SEM from six replicates. Different superscript letters indicate significant differences between experimental groups ($p < 0.05$).
ALA; a–linolenic acid, IVM; *in vitro* maturation, SEM; standard error of the mean.

Table 6. Effect of LA concentrations during IVM on the number of total cells and apoptotic cells produced by porcine blastocyst embryos *in vitro*

Concentration of LA	No. of examined blastocyst embryos	No. (mean) of total cells in blastocyst embryos	No. (mean) of apoptotic cells in blastocyst embryos
Control	25	40.8 ± 3.2[a]	1.5 ± 0.8[a]
10 μM	25	45.6 ± 3.4[b]	1.2 ± 1.1[b]
50 μM	25	41.4 ± 2.2[a]	1.5 ± 0.9[a]
100 μM	25	38.1 ± 3.9[a]	1.6 ± 1.1[a]

Results are presented as the mean ± SEM from five replicates. Different superscript letters indicate significant differences between experimental groups ($p < 0.05$).
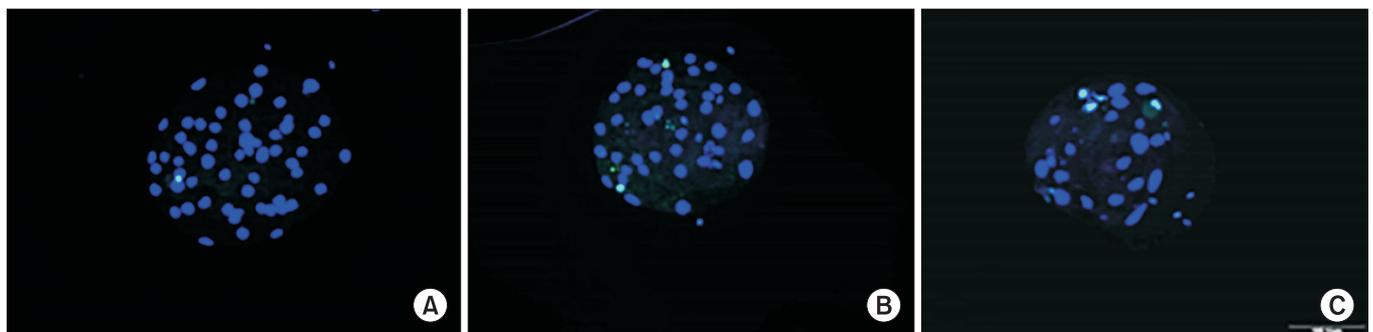LA; linoleic acid, IVM; *in vitro* maturation, SEM; standard error of the mean.

Fig. 3. Epifluorescent images of blastocyst embryos produced *in vitro* undergoing apoptosis. (A) Good quality, (B) normal quality, (C) bad quality. Chromatin content was assessed by DAPI staining (blue). TUNEL staining assessing DNA fragmentation (green). Scale bar = 100 μm. DAPI, 4′,6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling.
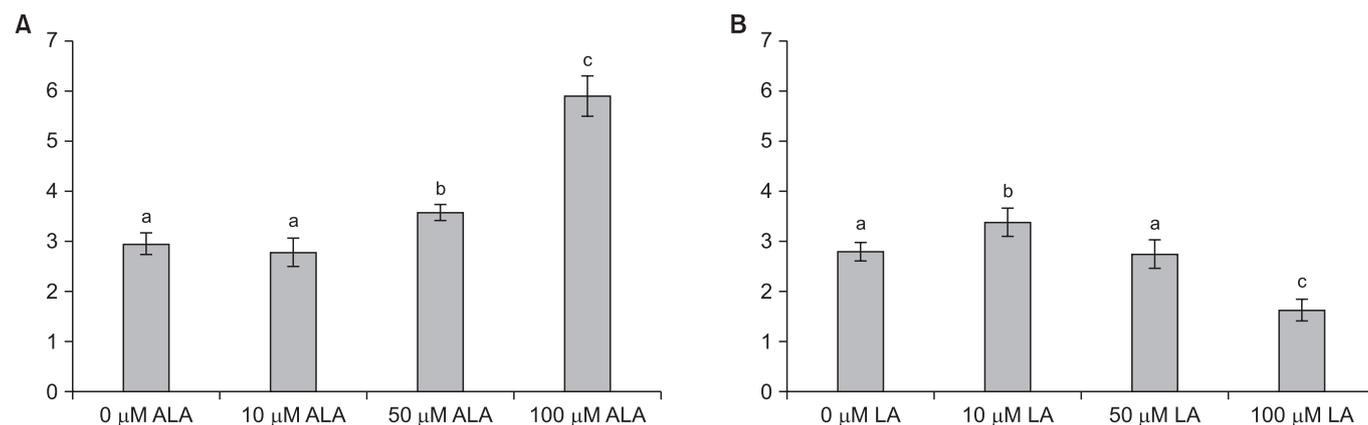
Fig. 4. Effect of ALA and LA concentrations on E2/P4-synthesis ratio during IVM of porcine follicular oocytes. E2/P4-synthesis ratio of COCs treated in the presence or absence of (A) ALA or (B) LA for 44 h. Results are presented as the mean ± SEM from 20 replicates. Different superscript letters indicate significant differences between experimental groups ($p < 0.05$). ALA; a-linolenic acid, E2; estrogen, IVM; *in vitro* maturation, LA; linoleic acid, P4; progesterone, SEM; standard error of the mean.

reduced the number of COCs containing fully expanded cumulus cells. These results were similar to our results described here. Moreover, our results indicated that 100 μM LA supplementation of maturation medium significantly inhibited (similar to ALA, but stronger) cumulus-cell expansion and oocyte nuclear maturation (Fig. 1B and Table 2). These results implied that the addition of high doses of LA also inhibited IVM of porcine COCs during IVM. Similar result was reported that LA significantly inhibits GV breakdown (35% in LA vs. 81% in control) in cumulus-free oocytes (Homa et al., 1992). Additionally, fatty acid-content analysis of the bovine follicular fluid collected from different-sized follicles showed significant decreases in LA concentration in large follicles as compared with small follicles. These results suggested that high LA concentrations could be a contributing factor to oocyte arrest at the GV stage. Subsequently, Marei et al. (2010) reported that LA supplementation to bovine oocytes during maturation alters the molecular mechanisms regulating oocyte maturation, resulting in a decreased percentage of oocytes at the MII stage and inhibition of cumulus-cell expansion in a dose-dependent manner. These inhibitory effects of ALA and LA supplementation may be related to changes in cell membrane fluidity and PGE$_2$ synthesis. First, PUFAs are necessary components of cell membranes; therefore, altering ALA and LA concentration can change membrane fluidity and affect processes mediated by the membrane due to the presence of unsaturated fatty acids with numerous double bonds, which cause hydrocarbon-chain bending and result in the formation of free spaces (Wiktorowska-Owczarek et al., 2015). In this context, inhibition of oocyte maturation by ALA and LA supplementation might be mediated by altering cumulus-cell membrane response to gonadotropic stimulation. In addition, ALA and LA serve as precursors for the synthesis of PGE$_2$, which plays an important role in cumulus-cell expansion and oocyte maturation (Marei et al., 2014). In mammals, LA can be transformed to DGLA and AA, and ALA can be transformed to EPA, and specifically, DGLA is a precursor for 1-series PG synthesis (PGF$_1$ or PGE$_1$), AA is a precursor for 2-series PG synthesis (PGF$_2$ or PGE$_2$), and EPA is a precursor for 3-series PG synthesis (PGF$_3$ or PGE$_3$) (Wathes et al., 2007).

In our porcine study (Fig. 2A), ALA supplementation (10 μM, 50 μM, and 100 μM) resulted in a dose-dependent decrease in PGE$_2$ synthesis in the spent medium during IVM. These results suggested that porcine COCs are capable of metabolizing ALA and moderating PGE$_2$ synthesis. A decrease in PGE$_2$ synthesis through the addition of high ALA concentrations during IVM could be associated with inhibited cumulus expansion and the decreased number of MII-stage oocytes (Fig. 1A and Table 1). As previously reported (Lands et al., 1992), PUFAs absorbed from the cellular environment are competitively synthesized into 1-, 2-, and 3- series PGs using a limited number of metabolic enzymes (especially desaturase and cyclooxygenase). This competition theory agrees with our results showing that high-dose ALA, which is a precursor of 3-series PGs, inhibited the synthesis of PGE$_2$, a 2-series PG. In sheep (Ghaffarilaleh et al., 2014) and bovine species (Marei et

al., 2009), COCs treated with ALA exhibit significantly increased PGE_2 concentrations as compared with the control group. In these studies, the conflicting results (when compared with our findings) might be species-specific. In our study, supplementation with 10 μM and 50 μM LA resulted in a marked increase in PGE_2 synthesis in the spent medium, whereas supplementation with 100 μM LA caused a significant decrease in PGE_2 synthesis (Fig. 2B). A decrease in PGE_2 synthesis following the addition of high LA concentrations during IVM could be associated with inhibited cumulus expansion and decreased numbers of MII-stage oocytes. In their bovine study, Marei et al. (2010) reported that LA supplementation (10 μM, 50 μM, and 100 μM) resulted in a dose-dependent increase in PGE_2 synthesis during IVM; however, increased PGE_2 synthesis could not overcome the inhibitory effect of LA on cumulus-cell expansion or oocyte maturation. The LA could be transformed to both DGLA (a precursor of 1-series PGs) and AA (a precursor of 2-series PGs) in mammals (Kinsella et al., 1990). When there is enough enzyme (desaturase and cyclooxygenase), added LA could be a precursor for both the 1- and 2-series PG, thus increased PGE_2 synthesis. However, when enzymes are limited, depending on the proportions of the various precursors present, 1-series PGs might be produced at the expense of 2-series PGs (Wathes et al., 2009). Therefore, in our porcine study, decreased PGE_2 synthesis following 100 μM LA supplementation might be explained by these interactions and competitive effects. These results also suggested that porcine COCs are capable of metabolizing LA and producing moderate amounts of PGE_2. In addition, decrease in PGE_2 synthesis below an appropriate level could be associated with inhibited cumulus expansion and decreased MII-stage progression of porcine oocytes matured in 100 μM LA. The above mentioned, relation to changes in cumulus-cell membrane fluidity and hormonal responsibility by addition PUFAs were also considered and needs further investigation.

Our results in porcine oocytes revealed no significant differences in cleavage or blastocyst rates between controls and 10 μM and 50 μM ALA-treated groups. However, oocytes matured in the presence of 100 μM ALA had significantly lower percentage of cleaved embryos and blastocyst rates. In addition, oocytes matured in the presence of 50 μM ALA showed higher-quality blastocyst embryos as demonstrated by higher cell number and decreased apoptosis (Tables 3 and 5; Fig. 3). In contrast to our findings, improved developmental rates to blastocyst-stage embryos were reported in bovine species (Marei et al., 2009), although significant increases in total cell number and decreased apoptosis were reported in both sheep (Ghaffarilaleh et al., 2014) and bovine species (Marei et al., 2009). In the bovine study (Marei et al., 2009), the authors suggested that these ALA-specific effects during IVM are mediated through both cyclic adenosine monophosphate and PGE_2 synthesis, whereas in sheep (Ghaffarilaleh et al., 2014), the authors suggest that these effects are associated with alterations in released reproductive hormones.

Little is known about the relation between changes in synthesis of these hormones (E2 and P4) in follicular fluid and production of good quality embryo from those follicles. However, near the time of ovulation, the phenomenon of prominent shift in the concentrations of E2 and P4 during the phase of final oocyte maturation (de Loos et al., 1991) indicate that the change of E2/P4-synthesis ratios is very important during maturation process. The finding that E2/P4 ratios in follicular fluid has been used as a surrogate marker of healthy and atretic follicles (Modina et al., 2014) supports this suggestion. High doses (100 μM) of ALA and LA treatment groups were excluded from the discussion, because they did not show normal maturation process such as abnormal cumulus cell expansion. In our study (Fig. 4A), the 50 μM ALA-treatment group showed higher E2/P4-synthesis ratios than the control group (3.59 ± 0.16 vs. 2.97 ± 0.22, respectively; $p < 0.05$). Moreover, oocytes matured in the presence of 10 μM LA resulted in better-quality blastocyst embryos in terms of higher cell number and decreased apoptosis (Table 4 and 6; Fig. 3), and this group showed a significantly higher E2/P4-synthesis ratio in spent medium relative to that observed in the control group (3.4 ± 0.28 vs. 2.81 ± 0.19; $p < 0.05$). These results are noteworthy and suggested the ability of porcine COCs to metabolize ALA and LA and moderate E2/P4 synthesis, with the optimal balance of hormone levels associated with the production of good-quality embryos. However, it is unclear whether these actions are mediated directly through the effect of PUFAs on E2/P4 synthesis or indirectly through altered PGE_2 secretion, because PGE_2 are thought act as luteotrophic factors in early luteal phase (Ghaffarilaleh et al., 2014) and affected E2 and P4 secretion (Wathes et al., 2007). Thus,

further investigations are needed to determine the association between PGE$_2$ secretion and E2 and P4 secretion.

CONCLUSION

In conclusion, the addition of optimal concentrations of ALA and LA to IVM medium for porcine oocytes resulted in physiological changes that increased *in vitro* production of high-quality blastocyst embryos exhibiting high cell number and decreased apoptosis. However, high doses of ALA and LA treatment results in detrimental effects. These results seem to be closely related to changes in PGE$_2$ synthesis and E2/P4-synthesis ratio. Further studies are required to determine the optimal application of fatty acid supplementation in porcine reproduction. These results can be used for both the establishment of an *in vitro* culture system for oocytes and for livestock-breeding programs through the use of an adequately balanced diet or supply of pharmacologic medications, as well as for basic research.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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