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Effect of Alpha Lipoic Acid on *in vitro* Maturation of Porcine Oocytes and Subsequent Embryonic Development after Parthenogenetic Activation[a]

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ABSTRACT

Alpha lipoic acid (ALA) is a biological membranes compound. As the antioxidant, it decreases the oxidized forms of other antioxidant substances such as vitamin C, vitamin E, and glutathione (GSH). To examine the effect of ALA on the *in vitro* maturation (IVM) of porcine oocytes, we investigated intracellular GSH and reactive oxygen species (ROS) levels, and subsequent embryonic development after parthenogenetic activation (PA). Intracellular GSH levels in oocytes treated with 50uM ALA increased significantly ($P < 0.05$) and exhibited a significant ($P < 0.05$) decrease in intracellular ROS levels compared with the control group. Oocytes matured with 50 uM of ALA during IVM displayed significantly higher cleavage rates (67.8% vs. 83.4%, respectively), and higher blastocyst formation rates and total cell number of blastocysts after PA (31.6%, 58.49 vs. 46.8%, 68.58, respectively) than the control group. In conclusion, these results suggest that treatment with ALA during IVM improves the cytoplasmic maturation of porcine oocytes by increasing the intracellular GSH levels, thereby decreasing the intracellular ROS levels and subsequent embryonic developmental potential of PA.

(Key Words : Antioxidant, Alpha lipoic acid, Porcine oocyte, *In vitro* maturation, Embryonic development)

INTRODUCTION

For many years, *in vitro* maturation (IVM) of oocytes was thought to be an important method in the treatment of infertility which is an issue of assisted reproduction techniques (ART) (Ali et al., 2006). But the increased oxygen concentration in the *in vitro* system than *in vivo* condition make higher reactive oxygen species (ROS) level in cells (Luvoni et al., 1996). Harmful factors that can be participated in the lower production of IVM, a higher level of ROS within the oocytes and culture medium lead to the oxidative stress that affects the *in vitro* system. Under optimal *in vivo* condition, a higher level of ROS was alleviated by both enzymatic and non-enzymatic antioxidants. Supplementing maturation medium with different antioxidants has been reported that overcome the oxidative stress and improves oocyte developmental competence (Combelles et al., 2009).

Alpha lipoic acid (ALA) is not only component of biological membranes and but also an imperative cofactor of mitochondrial dehydrogenases that well known for its antioxidative properties (Packer et al., 1995). ALA as a biological antioxidant, decrease the oxidized forms of other antioxidant substances such as vitamin C, vitamin E, and glutathione (GSH) (Golbidi et al., 2011). ALA has been found to be a therapeutic agent for many chronic diseases such as diabetes mellitus and its complications, hypertension, Alzheimer's disease, Down syndrome, cognitive dysfunction and some types of cancers (Gomes and Negrato, 2014). Many signaling transduction pathways are regulated by ALA. One of those, nuclear factor and also a transcription factor, erythroid 2-related factor (*Nrf2*) is highly sensitive to oxidative stress (Chen et al., 2015). *Nrf-2* transcript levels were significantly increased in oocytes treated with another antioxidant carboxyethylgermanium sesquioxide than those in the control group (Kim et al., 2015). Addition of ALA to cadmium-treated cells reactivated *Nrf2* and regenerated GSH through elevating the *Nrf2*-downstream genes

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gamma-glutamate-cysteine ligase (*ɣ-GCL*) and glutathione disulfide reductase (*GSR*), both of which are key enzymes for GSH synthesis (Zhang et al., 2017). Presence of ALA-induced significant higher developmental rate of secondary preantral follicles in bovine, and follicles, oocytes, and embryos in mouse (Talebi et al., 2012; Zoheir et al., 2017).

GSH is a tripeptide consist of glutamate, cysteine, and glycine. It is included in the organs of all mammalians, particularly higher in the liver. The decline of intracellular GSH levels has been demonstrated to be related to cancer, Alzheimer's disease, cystic fibrosis, and HIV (Ballatori et al., 2009; Saing et al., 2016). The increase of *Nrf2*-mediated transcription which was upregulated by ALA decreased the level of GSH in rat liver (Suh et al., 2004). *ɣ-GCL* and *GSR* which are related to ALA, participate in GSH synthesis. *ɣ-GCL* is composed of *GCLM, GCLC* which are genetically different but both are important for GSH synthesis (Yang et al., 2005). *GSR* decreases *GSSG* in an NADPH-dependent reaction that makes more *GSH* (Yao et al., 2006). Excessed expression of *Bax* triggers apoptotic death caused by cytokine deprivation in IL-3-dependent cell line. *Bcl-2* expression which decreases cell death is downregulated by excessed expression of *Bax* (Oltval et al., 1993).

Despite numerous reports presenting the effect of ALA, there are not enough data available about oocyte development and blastocyst formation under the effect of ALA, the study of their effects on porcine oocytes is required. The main purpose of the present study was to investigate whether ALA upregulates GSH and downregulate ROS in individual ALA levels, embryonic competence after parthenogenesis (PA). This study will provide more information on the role of antioxidants and GSH in porcine embryo by ALA.

MATERIALS AND METHODS

1. Chemicals

All chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) unless otherwise stated. Alpha lipoic acid stock (1 mg/ml) was prepared in 0.5% dimethyl sulphoxide (DMSO) (w/v). The working solution was diluted with culture medium (TCM199; Invitrogen Corporation, Carlsbad, CA, USA) so that the final concentrations of DMSO in the culture medium were not more than 0.01% (v/v). 0.01% DMSO was used as a sham control.

2. Oocyte collection and *in vitro* maturation

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in normal saline supplemented with 100 IU/L penicillin G, and 100 mg/ml streptomycin sulfate maintained at 32 to 35°C within two h of collection. The cumulus-oocyte complexes (COCs) were aspirated from 3- to 6- mm in diameter superficial follicles with an 18-gauge needle and washed with HEPES-buffered Tyrode's medium (TLH) containing 0.05% (w/v) polyvinyl alcohol (TLH-PVA). Next, only COCs having ≥ 3 uniform layers of compact cumulus cells and homogenous cytoplasm were selected, and 60 COCs were placed into each well of a four-well Nunc dish (Nunc, Roskilde, Denmark) containing 500 μl of TCM199, which was treated with 0.6 mM of cysteine, 0.91 mM of sodium pyruvate, 10 ng/ml of epidermal growth factor, 75 μg/ml of kanamycin, 10% (v/v) of porcine follicular fluid (pFF), 10 IU/ml of equine chronic gonadotropin (eCG), and 10 IU/ml of hCG (Intervet, Boxmeer, Netherland). The selected COCs were maturated with hormones from 22 to 23 h and without hormones from 18-19 h at 39°C in 5% CO_2 in 95% humidified air. The COCs during IVM were treated with concentration (0, 10, 50 and 100 μM) of ALA according to the experimental design.

3. Measurement of intracellular GSH and ROS levels

Measurement of intracellular GSH and ROS levels After IVM, the COCs were sampled 40–42 hours after IVM to evaluate intracellular GSH and ROS levels. Evaluation of the GSH and ROS levels was performed as previously described methods (Abeydeera et al., 1998; Meister, 1983). Briefly, 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CellTracker Blue; CMF2HC; Invitrogen Corp.) and 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen Corp., Paris, France) were used to detect intracellular GSH (blue fluorescence) and ROS levels (green fluorescence), respectively. From each treatment group, ten oocytes were incubated in the dark for 30 minutes in TLH-PVA supplemented with 10 μmol/L CellTracker Blue and 10 μmol/L H2DCFDA. After incubation, the oocytes were washed with TLH-PVA, and the fluorescence was evaluated using an epifluorescence microscope (TE300; Nikon Corp.) with UV filters (370 nm for GSH and 460 nm for ROS) at

200 x magnification. The fluorescence intensities of the oocytes were analyzed using Adobe Photoshop software (Version CS6; San Jose, CA, USA). The experiment was repeated three times (GSH samples, N = 30; ROS samples, n = 30).

4. Parthenogenetic activation of oocytes

For parthenogenetic activation, the COCs after IVM were denuded by softly pipetting with 0.1% hyaluronidase, washed three times with TLH-PVA and then rinsed twice in activation medium consisting of 280 mM mannitol solution, 0.01 mM $CaCl_2$, and 0.05 mM $MgCl_2$. For activation, the matured oocytes (at MII stage) were placed between electrodes covered with activation medium in a chamber attached to an electrical pulsing machine (LF101; NepaGene, Chiba, Japan). Oocytes were activated with two direct-current pulses of 120 V/mm for 60 μsec. After electrical activation, oocytes were immediately moved into IVC medium supplemented with 5 μg/ml cytochalasin B for 6 h. The PA embryos were washed three times in fresh IVC medium, aliquoted into 30 μl IVC droplets (10 gametes per drop), covered with pre-warmed mineral oil, and then cultured at 39°C under 5% O_2, 5% CO_2, and 90% N_2 humidified atmosphere for 7 days.

5. *In vitro* embryo culture

The PA embryos were rinsed three times with IVC medium (porcine zygote medium 3) and cultured in 30-μL microdrops of IVC medium. Embryos in culture medium were covered with pre-warmed mineral oil and incubated at 39°C for seven days under a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2.

6. Embryo evaluation and total cell count of blastocysts

The day of PA was considered Day 0. The embryos were estimated for cleavage using a stereomicroscope on Day 2 (48 h). Cleaved embryos were classified into three different groups (2 to 3, 4 to 5, and 6 to 8 cells). Blastocyst formation was evaluated on Day 7 (168 h) after PA, and blastocysts were classified by the degree of expansion and hatching status as follows: early blastocyst (small blastocyst with a blastocoel equal to or less than half of the embryo volume), expanded blastocyst (a large blastocyst with a blastocoel greater than half of the embryo volume or a blastocyst with a blastocoel completely filling the embryo), and hatched blastocyst (hatching or already hatched blastocyst). For counting the total cell number of blastocysts, at Day 7, the blastocysts were collected and washed in 1% (w/v) PBS-BSA and stained with 5μg/mL Hoechst-33342 (bisbenzimide) for 5 min. After a final wash in PBS-BSA, the embryos were fixed in 0.1% paraformaldehyde in PBS. Next, the blastocysts were put on slide glasses in a drop of 100% glycerol, covered softly with a cover slip, and detected using a fluorescence microscope (Nikon Corp., Tokyo, Japan) at ×400 magnification. The experiment was repeated three times. The total number of oocytes used per treatment group is presented in Table 2.

7. Gene expression analysis via quantitative real-time polymerase chain reaction

For the gene expression study, 120 mature oocytes and cumulus cells which was defined as the optimal concentration at parthenogenetic activation (50μM of Alpha lipoic acid) were separately sampled using a stereomicroscope. All samples were frozen at -80 °C until analyzed. The expression levels of *Bcl-2, NQO-1, Nrf-2, Keap1, GCLC, GCLM,* and, *GSR* mRNA in the oocytes and cumulus cells were investigated via quantitative RT-PCR. In brief, total RNA was extracted using TRIzol reagent (Invitrogen Corporation), and the total RNA concentration was evaluated by measurement of the absorbance at 260 nm. First-strand complementary DNA (cDNA) was made by subjecting 1 mg of total RNA to reverse transcription using Moloney murine leukemia virus reverse transcriptase (Invitrogen Corporation) and random primers (9 mers; TaKaRa Bio Inc.). To analysis the conditions for the logarithmic-phase PCR amplification of target mRNA, 1 mg aliquots were amplified using differing numbers of cycles. The housekeeping gene *18S* was amplified to rule out the possibility of RNA degeneration and to control for the variation in mRNA concentrations in the reverse transcription reaction. *18S* and the target genes were quantified using 40 cycles. The cDNA was amplified in a 20-mL PCR reaction composed of 1 U Taq polymerase (Intron BioTechnologies, Co., Ltd., Seongnam, Korea), 2-mM deoxyribonucleoside triphosphates mix, and 10 pM of each gene-specific primer. The quantitative RT-PCR was carried out with 1-mL cDNA template added to 10 μl of 2x SYBR Premix Ex Taq (TaKaRa Bio, Inc.) containing specific primers at a concentration of 10 pM each. The reactions were performed at 40 cycles, and the cycling parameters were as follows: denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds. All

oligonucleotide primer sequences are presented in Table 1. During the end of the extension phase of each cycle, the fluorescence intensity was estimated. The threshold value for the fluorescence intensity of all samples was set according to manual. The reaction cycle at which the PCR products exceeded this fluorescence intensity threshold was considered the threshold cycle (Ct) in the exponential phase of the PCR amplification. The expression of each target gene was quantified compare to that of the housekeeping gene (*18S*). The relative quantification was based on a comparison of Ct at constant fluorescence intensity. The amount of transcript present was inversely related to the observed Ct and, for every twofold dilution in the amount of transcript, the Ct was expected to increase by one. The relative expression (R) was calculated using the following equation: $R= 2^{-[Ct\ sample\ -\ Ct\ control]}$. To determine a normalized arbitrary value for each gene, every obtained value was normalized to that of *18S*. The experiments were repeated at least three times.

8. Experimental design

In experiment 1, the effect of alpha lipoic acid treatment with various concentration (0, 10, 50, 100 μM) during IVM on intracellular levels of GSH and ROS was investigated. In experiment 2, the effect of the ALA treatment during IVM on the subsequent embryonic development of PA was examined. In experiment 3, the effect of ALA treatment on the mRNA expression of (*Bcl2, Keap1, Nrf2, GCLC, GCLM, GSR, NQO1*) in MII oocytes and cumulus cells were analyzed.

9. Statistical analysis

The statistical analyses were conducted using SPSS 21.0 (SPSS, Inc., Chicago, IL, USA). Percentage data (e.g., rates of maturation, cleavage, blastocyst formation) was compared using one-way ANOVA followed by Duncan's multiple range tests. All results are expressed as the mean±standard error of the mean. Probability values less than 0.05 were considered to be statistically significant unless otherwise stated.

RESULTS

1. Effect of Alpha lipoic acid on oocyte maturation during IVM

We performed porcine IVM supplemented with various concentration of ALA (0, 10, 50, and 100μM). After 44hours, we researched the maturation of porcine MII oocyte by evaluating intracellular GSH and ROS levels derived from the maturation medium supplemented with ALA after IVM. The group of 50 μM of ALA treatment showed significantly increased ($P < 0.05$) intracellular GSH levels and the intracellular ROS levels significantly decreased compared to the control group (Fig. 1).

Table 1. Primers used for gene expression analysis.

Gene name	Primer sequence	Product size (bp*)	Genbank accession number or reference
Keap1	F: 5'-AGCTGGGATGCCTCAGTGTT-3' R: 3'-AGGCAAGTTCTCCCAGACATTC-3'	100	NM001114671.1
GSR	F: 5'-CAGATTGCAAAGACTGCAAA-3' R: 5'-TCTGAAAGCAAAGAATCACTCC-3'	207	XM_003483635_4
Bcl-2	F: 5'-AATGACCACCTAGAGCCTTG-3' R: 5'-GGTCATTTCCGACTGAAGAG-3'	193	NM_214285
GCLC	F: 5'-GTTTTGTGAATCAGGACCCTA-3' R: 5'-GCTTAGCTGAAGCTTTATTGC-3'	212	XM_003483635_4
GCLM	F: 5'-TGATATTGGCTAGCAGTTCACC-3' R: 5'-TAGCCTGCCACCCACTTTAT-3'	195	XM_001926378.4
Nrf2	F: 5'-CCCATTCACAAAAGACAAACATTC-3' R: 3'-GCTTTTGCCCTTAGCTCATCTC-5'	71	Nannelli A et al., 2009
NQO1	F: 5'-CTAGAGAGCACCCAACTCCT-3' R: 5'-GGCGTAATTAGTCACTGGGTA-3'	191	NM_001159613.1
18S	F: 5'-CGCGGTTCTATTTTGTTGGT-3' R: 5'-AGTCGGCATCGTTTATGGTC-3'	227	NR_046261.1

*bp: base pares

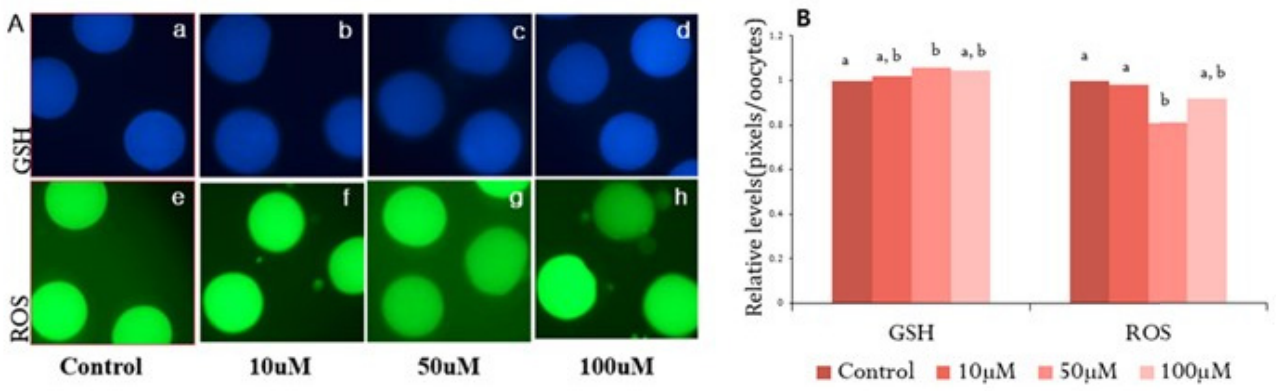

Figure 1. Epifluorescent photomicrographic images of in vitro-matured porcine oocytes. (A) Oocytes were stained with CellTracker Blue (a-d) and 20,70-dichlorodihydrofluorescein diacetate (e-h) to detect the intracellular levels of glutathione (GSH) and reactive oxygen species (ROS), respectively. Metaphase II oocytes derived from the maturation medium supplemented with 0uM of alpha lipoic acid (ALA; a and e), ten μM of ALA (b and f), 50 μM of ALA (c and g), and 100 μM of ALA (d and h). (B) The relative levels of intracellular GSH and ROS in IVM porcine oocytes among the four groups (0 μM, ten μM, 50 μM, 100 μM ALA). Within each group end point (GSH and ROS), the bars with different letters (a-b) are significantly (P < 0.05) different. A total number of examined oocytes: GSH samples, N= 30; ROS samples, N= 30. The experiment was replicated three times.

2. Effect of ALA supplemented to IVM media on subsequent embryonic development after PA

MII oocytes from each IVM groups were performed PA. After PA we found that the cleavages rate of zygotes was significantly increased (P<0.05) in the 50 μM of ALA treatment than control groups (Table 2, Fig. 2). Embryonic developmental competence to the blastocyst stage and total cell numbers in blastocyst after PA with ALA treatment showed an increase compared to control groups (Table 2). The embryos treated with 50 μM of ALA suggested the highest (P < 0.05) blastocyst formation rates (46.8%) and total cell numbers in blastocysts (68.6) compared with the other groups (Fig. 3).

3. Effect of ALA treatment during IVM on gene expression in oocytes and cumulus cells

The effects of ALA on the expression was investigated about antioxidative effect and apoptosis-related genes, we performed real-time PCR to evaluate the mRNA expression levels of *GCLC, GCLM, Keap1, Bcl-2, GSR, NRF2* and *NQO1* in the oocytes and the cumulus cells of control group and treated with 50 μM of ALA which was thought to be optimal concentration in previous experiments. Keap1 gene expression is significantly decreased in cumulus cells treated ALA group compared with control groups (P < 0.05). Also, *Nrf2, GSR* gene expression is significantly higher in both oocytes and cumulus cells at treated groups (P < 0.05). There were no significant differences in *Bcl2, GCLC, GCLM* and *NQO1* in oocytes and cumulus cells and *Keap1* in oocytes (Fig. 4).

Table 2. Effect of ALA treatment during IVM on the embryonic development after parthenogenetic activation (PA)

Concentration of ALA (uM)	No. of embryos cultured for PA (3*)	No. (%) of embryos developed to ≧ 2-cell	No. (%) of embryos developed to Blastocyst	Total cell number (mean±SEM)
0	169	114(67.80±3.48)^{a}	54(31.59±3.83)^{a}	20(58.49±2.29)^{a}
10	165	119(72.17±0.82)^{a}	65(38.62±4.22)$^{a, b}$	22(62.36±2.06)$^{a, b}$
100	171	142(83.47±2.15)^{b}	81(46.75±4.55)^{b}	21(68.58±2.34)^{b}
200	172	129(75.22±3.54)$^{a, b}$	66(37.80±5.51)$^{a, b}$	21(61.18±2.26)$^{a, b}$

a-b Values with different superscripts with a column differ significantly (P<0.05)
The experiment is repeated three times. The data represent means±SEM.

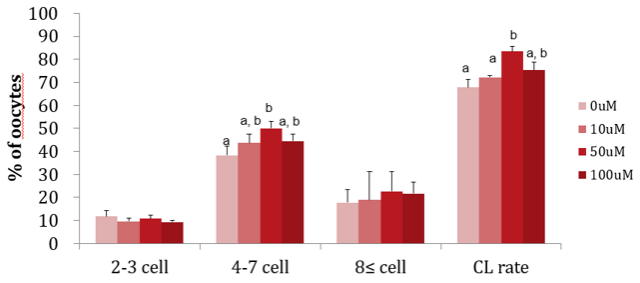

Figure 2. Effect of ALA treatment during IVM on the cleavage pattern in embryos produced by PA and evaluation of cleavage rate at Day 2. Within each end point, bars with different letters (a, b) are significantly (P<0.05) different for different IVM type. CL: Cleavage

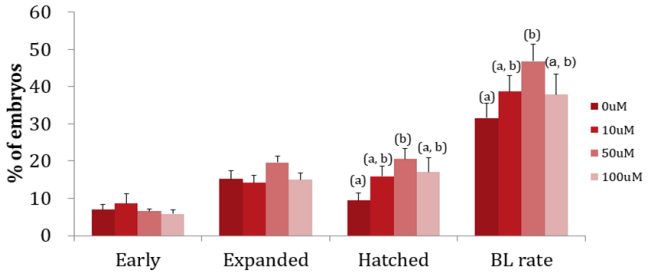

Figure 3. Effect of ALA treatment during IVM on the cleavage pattern in embryos produced by PA and blastocyst formation at Day 7. Within each end point, bars with different letters (a, b) are significantly (P<0.05) different for different IVM type. BL: blastocyst

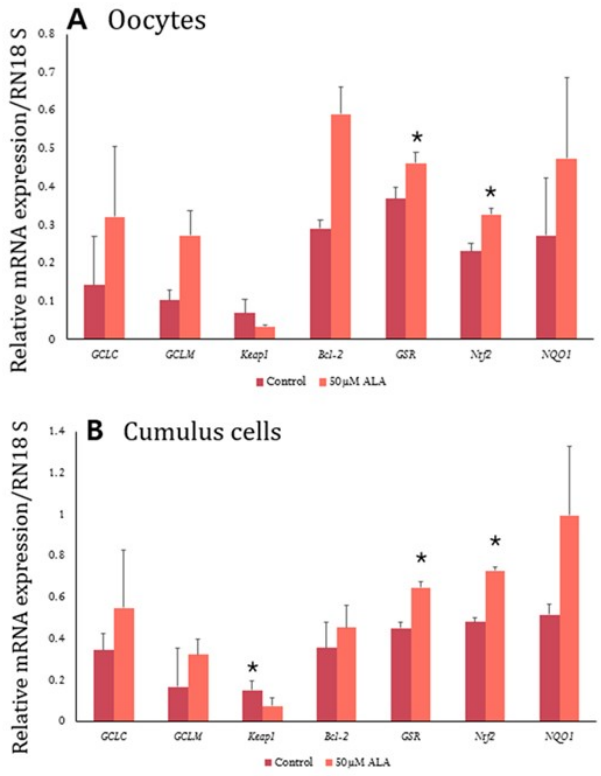

Figure 4. mRNA expression levels (Mean ± SEM) of *GCLC*, *GCLM*, *KEAP1*, *BCL2*, *GSR*, *NRF2* and *NQO1* in oocytes (A) and cumulus cells (B) treated with ALA during IVM. The experiment was replicated three times.

DISCUSSION

For several years, researchers have tried to improve the IVM of oocytes. It has been that IVM system is suboptimal condition compared to *in vivo*. The main cause is environmental differences between *in vivo* and *in vitro* conditions that occur the accumulation of ROS (Coy and Romar, 2002; Funahashi and Day, 1997). In pigs, it is especially difficult to obtain high rates of blastocyst development *in vitro* condition (Gil et al., 2010). Therefore, many antioxidants have been used to prevent the accumulation of oxidative stress by a supplement in cultivation medium (Abedelahi et al., 2010; Ali et al., 2003; Talebi et al., 2012). However, the advantages of adding antioxidants in oocyte maturation medium remain controversial (Combelles et al., 2009). Oocyte maturation is a key factor in IVM systems because oocyte maturation affects early embryonic development and survival, fetal growth and subsequent events (Eppig and O'Brien, 1998; Mtango et al., 2008; Sirard et al., 2006). Therefore, ALA was supplemented to improve the maturation of porcine oocytes *in vitro* maturation.

To determine the status of oocyte cytoplasmic maturation, we investigated the level of intracellular GSH as a molecular marker. GSH is related to various cellular processes that reduce oxidative stress (Luberda, 2005; Meister, 1983). One of those, we focused on *Nrf-2* pathway and relationship with ALA and GSH. ALA upregulated GSH via activating *Nrf2* signaling pathway in aged rats (Suh et al., 2004). ALA-treated HepG2 cells show increased levels of mRNA and protein of GCLC, GCLM, and GSR, which means upregulation of

intracellular GSH levels via activating Nrf2 signaling pathway (Zhang et al., 2017). In hfRPE cells, the mRNA and the protein levels of ɣ-GCL subunits GCLC and GCLM were increased by 0.5 mM ALA (Volobоueva et al., 2005) and ALA dose-dependently upregulated the mRNA and the protein levels of GSR (Ide, 2014). ROS in oocytes is a key parameter to evaluate oocyte status (Halliwell and Aruoma, 1991; Yang et al., 1998). In this study, Intracellular GSH levels were significantly upregulated and intracellular ROS levels were downregulated in porcine oocyte maturation by 50 μM of ALA. Furthermore, we investigated mRNA expression of the *Nrf-2, GSR, GCLM,* and *GCLC* in porcine oocytes and cumulus cells. We could find a significant increase in the mRNA expression of *Nrf-2* and *GSR* in 50 μM treated ALA group than control group. However, there was no significant difference in *GCLC* and *GCLM*.

Because of the beneficial effect of ALA on cytoplasmic maturation, embryonic development and blastocyst viability after PA were also improved by 50 μM ALA treatment. Previous studies suggested the beneficial effect of ALA on mouse isolated preantral follicles by decreasing ROS concentration the culture period and on bovine secondary preantral follicles development and growth (Talebi et al., 2012; Zoheir et al., 2017). Compare to our study, the optimal concentration of ALA is quite different. Our results indicate that 50 μM ALA significantly increased porcine blastocyst formation rates after PA different with 100 μM in the mouse. The difference might be derived from the different species, the composition of medium or status of the oocyte when treated. In conclusion, ALA treatment during IVM was beneficial for cytoplasmic maturation of porcine oocytes by increasing intracellular GSH levels, thereby decreasing ROS concentrations. Furthermore, porcine oocytes treated with 50 μM ALA may have increasing developmental competence, which would greatly increase blastocyst formation in PA derived embryos. Therefore, our results suggest that ALA improves the quality of porcine oocytes and subsequent in vitro development when 50 μM ALA is supplemented.

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