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The Beneficial Effects of Ferulic Acid supplementation during *In Vitro* Maturation of Porcine Oocytes on Their Parthenogenetic Development[a]

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ABSTRACT

Ferulic Acid (FA) is a metabolite of phenylalanine and tyrosine, a phenolic compound commonly found in fruits and vegetables. Several studies have shown that FA has various functions such as antioxidant effect, prevention of cell damage from irradiation, protection from cell damage caused by oxygen deficiency, anti-inflammatory action, anti-aging action, liver protective effect and anti-cancer action. In this study, we investigated the maturation rate, intracellular glutathione (GSH) and reactive oxygen species (ROS) of porcine oocytes by adding FA to the in vitro maturation (IVM) medium and examined subsequent embryonic developmental competence at 5% oxygen through parthenogenesis. There is no significant difference between the control group (0μM) and treatment groups (5μM, 10μM, 20μM) on maturation rates. Intracellular GSH levels in oocyte treated with 5μM of FA significantly increased ($P < 0.05$), and 20μM of FA revealed significant decrease ($P < 0.05$) in intracellular ROS levels compared with the control group. Oocytes treated with FA exhibited significantly higher cleavage rates (79.01% vs 89.19%, 92.20%, 90.89%, respectively) than the control group. Oocytes treated with 10μM showed significantly higher blastocyst formation rates (28.3% vs 40.3%, respectively) after PA than the control group. Total cell numbers in blastocyst of 10μM FA displayed significantly higher (39.4 vs 51.9, respectively) than the control group. In conclusion, these results suggested that treatment with FA during IVM improved the developmental potential of porcine embryos by increasing intracellular GSH synthesis and reducing ROS levels. Also, there was an improvement of cleavage rate, blastocyst formation and total cell numbers in blastocysts. It might be associated with Keap1-Nrf2 pathway as an antioxidant regulate pathway that plays a crucial role in determining the sensitivity of cells to oxidative damages by regulating the basal and inducible expression of enzymes which is related to detoxification and anti-oxidative effects, stress response enzymes and/or proteins and ABC transporters.

(Keywords : Ferulic Acid, Antioxidant, Porcine oocyte, IVM, PA, Keap1-Nrf2 pathway)

INTRODUCTION

With the development of science and technology, a wide range of research and development has become possible through assisted reproductive technology. For example, with increasing infertility rates in modern society, childbirth through assisted reproductive technology has become possible (Sunderum, et al. 2013) . In addition, assisted reproductive technologies are being used to develop superior breed of cattle in the field of livestock farming (Mapletoft and Hasler 2005). Furthermore, *in vitro* maturation (IVM) techniques have been used to study embryo development. However, despite the advances of assisted reproductive technology, low efficiency due to low maturation rates of oocytes is still a problem. One of the causes of low maturation rates is excessive oxygen exposure (Agarwal, et al. 2006). When oxygen is present, an intermediate product, Reactive Oxygen Species (ROS), is produced, which acts as a strong oxidizing agent. There are superoxide anion, hydrogen peroxide, and hydroxyl radicals, which can induce structural and functional changes, damaging the gametes or embryos (Combelles, et al. 2009).

In the body environment, ROS is produced at low

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concentrations in the genital tracts (Agarwal, et al. 2003). There are various anti-oxidative enzymes in vivo such as calcium oxide, superoxide dismutase, glutathione peroxidase and antioxidants such as vitamin C, vitamin E, vitamin A, pyruvate, glutathione, taurine, and hypotaurine (Agarwal, et al. 2005). The defense system of this body stabilizes the ratio of ROS under physiological conditions (Agarwal, Saleh and Bedaiwy 2003, Agarwal, et al. 2005). Also, the oxygen concentration in the body is about 5%, so that the reproductive organ is exposed to very low oxygen conditions as compared with the in vitro environment. *In vitro* maturation techniques are exposed to atmospheric oxygen (20% O_2), which is high compared to the body environment. Thus, during manipulation for assisted reproductive technology, exposure to high concentrations of oxygen is inevitable, which causes more ROS production and is thus one of the major causes of oxidative damage to the oocyte.

Ferulic acid (FA) is a metabolite of phenylalanine and tyrosine, a phenolic compound commonly found in fruits and vegetables. FA has a benzene ring and unsaturated residues, and the benzene ring can terminate the free radical chain reaction. In addition, the carboxylic acid group of FA can prevent free radicals from damaging the cell membrane. Also, the carboxylic acid group acts to protect the cell membrane from lipid peroxidation by binding to the lipid bilayer of the cell membrane. When absorbed into the body, FA is more prolonged in the blood than antioxidants such as vitamin C, allowing longer free radical scavenging (Srinivasan, et al. 2007). Several studies have shown that FA has various functions such as anti-oxidative effect (Ogiwara, et al. 2002), prevention of cell damage from irradiation (Seawright, et al. 2017), protection from cell damage caused by oxygen deficiency (Chen, et al. 2017), anti-inflammatory action, anti-aging action, liver protective effect (Rukkumani, et al. 2004) and anti- colon carcinogenesis (Kawabata, et al. 2000). Various studies using FA have been carried out, but studies using mammalian germ cells have not yet been conducted. In this study, to overcome the low efficiency of IVM by ROS, we investigated the maturation rate, GSH and ROS of porcine oocytes by adding FA to the IVM medium and examined embryonic developmental competence at 5% oxygen after embryo formation through parthenogenesis.

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. FA stock (200μM) was prepared in 0.5% dimethyl sulphoxide (DMSO) (w/v). The working solution was diluted with TC-M199 (TCM199; Invitrogen Corporation, Carlsbad, CA, USA) media so that the final concentrations of DMSO in the culture medium were not more than 0.01% (v/v) and 0.01% of DMSO was used as a sham control.

2. Oocyte collection and *in vitro* maturation (IVM)

Ovaries of prepubertal gilts were collected at a local slaughter house and transported to the laboratory within 2 hours in normal saline (0.9% of NaCl, w/v) solution supplemented with 100 IU/L penicillin G and 100 mg/mL streptomycin sulfate at 32 °C to 35 °C. The cumulus oocyte complexes (COCs) in the porcine ovaries were aspirated from 3- to 6- mm diameter superficial follicles using an 18-gauge needle attached to a 10- mL disposable syringe and allowed to settle in 15-mL conical tubes at 37 °C for 10 min. The supernatant was discarded, and the precipitate was resuspended with HEPES-buffered Tyrode's medium (TLH) containing 0.05% (w/v) polyvinyl alcohol (TLH-PVA). The precipitate was investigated using a stereomicroscope to pick up the COCs. Only COCs with more than 3 homogenous layers of compact cumulus cells and uniform cytoplasm were selected and washed three times in TLH- PVA. Approximately 60 COCs were placed into each well of a four-well Nunc dish (Nunc, Roskilde, Denmark) containing 500 μL of culture medium (TCM199; Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 0.6 mM cysteine, 0.91 mM sodium pyruvate, 10 ng/mL epidermal growth factor, 75μg/mL kanamycin, 1 μg/mL insulin, 10% (vol/vol) porcine follicular fluid (pFF), 10 IU/mL equine chronic gonadotropin (eCG), and 10 IU/mL hCG (Intervet, Boxmeer, Netherlands). The selected COCs were cultured at 39 °C with 5% CO_2 in a 95% humidified chamber for IVM. After 21 to 22 h of maturation with hormones, the COCs were washed twice in fresh hormone-free IVM medium and then incubated in hormone-free IVM medium for an additional 21 to 22 h. The COCs were treated with or without FA during the entire maturation time.

3. Measurement of intracellular GSH and ROS levels

The oocytes at 42 - 44 h after IVM were sampled to determine intracellular GSH and ROS levels. GSH and ROS level assessment was carried out as described (You, et al. 2010, Nasr-Esfahani, et al. 1990). Briefly, 2', 7'-dichlorodihydro-fluorescein diacetate (H2DCFDA, Invitrogen Corporation) and 4-chloromethyl-6.8-difluoro-7-hydroxycoumarin (Cell Tracker Blue, CMF2HC; Invitrogen Corporation) were used to detect intracellular ROS levels (green fluorescence) and GSH levels (blue fluorescence), respectively. Ten oocytes from each treatment group were incubated (in the dark) for 30 min in TLH-PVA supplemented with 10 μM H2DCFDA and 10 μM Cell Tracker Blue. After incubation, the oocytes were washed with Dulbecco's phosphate buffered saline (Invitrogen Corporation) containing 0.1% (w/v) PVA, placed into 10-μL droplets, and fluorescence was evaluated using an epifluorescence microscope (TE300; Nikon, Tokyo, Japan) with UV filters (460 nm for ROS and 370 nm for GSH). These fluorescent images were saved as graphic files in TIFF format. The fluorescence intensity of the oocytes was analyzed using Image J software (Version 1.41; National Institutes of Health, Bethesda, MD, USA) and normalized to control oocytes. The experiment was repeated three times (GSH samples, N=30; ROS samples, N=30).

4. Parthenogenetic activation of oocytes

For parthenogenetic activation (PA), the COCs 42 – 44 h after IVM were denuded by gently pipetting with 0.1% hyaluronidase, washed three times in TLH-PVA and then rinsed twice in activation medium (280 mM mannitol solution containing 0.01 mM $CaCl_2$ and 0.05 mM $MgCl_2$). For activation, the MII stage oocytes were placed between electrodes covered with activation medium in a chamber connected to an electrical pulsing machine (LF101; Nepa Gene, Chiba, Japan). Oocytes were activated with two direct-current (DC) pulses of 120 V/mm for 60 μsec. After electrical activation, oocytes were immediately placed in IVC medium supplemented with 5 μg/mL cytochalasin B for 4 h. The PA embryos were washed three times in fresh IVC medium, placed in 25 μL IVC droplets (10 gametes per drop) covered with pre-warmed mineral oil, and then cultured at 39 °C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2 for 7 days. The experiment was repeated three times. The total number of oocytes used per treatment group is presented in Table 1.

5. *In vitro* culture (IVC)

The PA embryos were washed 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 7 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 evaluated for cleavage using a stereomicroscope on Day 2 (48 h). Evenly cleaved embryos were classified into three groups (2 to 3, 4to 5, and 6 to 8 cells). Blastocyst formation was assessed on Day 7 (168 h) after PA, and blastocysts were classified according to 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

Table 1. Effect of FA treatmentduring IVM on the embryonic development after parthenogenetic activation (PA).

Concentration of FA (μM)	No. of embryos cultured	No. of embryos developed to (%*)		Total cell numbers in blastocyst (N)†
		≧ 2-cell	Blastocyst	
0	140	110(79.0±2.2)[a]	39(28.3±2.6)[a]	39.4±2.5[a](43)
5	140	125(89.2±2.3)[b]	47(33.8±3.0)[a,b]	45.0±2.3[a,b](53)
10	132	122(92.2±3.8)[b]	53(40.3±1.4)[b]	51.9±2.5[b](41)
20	133	121(90.9±2.9)[b]	50(36.3±3.0)[a,b]	45.9±2.4[a,b](41)

* Percentage of total cultured oocytes

† Number of examined blastocysts

[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.

(hatching or already hatched blastocyst). To quantify the total cell number of blastocysts, at Day 7, the blastocysts were collected and washed in 1% (w/v) of PBS-BSA and stained with 5μg/mL Hoechst-33342 (bisbenzimide) for 5 min. After final wash in PBS-BSA, the embryos were fixed briefly in 0.1% paraformaldehyde in PBS. Next, the blastocysts were mounted on glass slides in a drop of 100% glycerol, covered gently with a cover slip, and observed using a fluorescence microscope (Nickon 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 1.

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

For the gene expression study, 160 of matured oocytes and cumulus cells which concentration is the most effective at PA (10 μM of FA) were separately sampled using a stereomicroscope. All samples were stored at - 80 °C until analyzed. For the reveal of anti-oxidative pathways, *Keap1, Bax, NQO-1, Nrf-2, GCLC, GCLM,* and, *GSR* mRNA gene expression levels in the oocytes and cumulus cells were analyzed via quantitative RT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen Corporation) according to the manufacturer's protocol, and the total RNA concentration was determined by measuring the absorbance at 260 nm. First-strand complementary DNA (cDNA) was prepared 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., Otsu, Shiga, Japan). To determine 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 PCR amplified to rule out the possibility of RNA degradation and to control for the variation in mRNA concentrations in the reverse transcription reaction. A linear relationship between PCR product band visibility and the number of amplification cycles was observed for the target mRNAs. 18S and the target genes were quantified using 40 cycles. The cDNA was amplified in a 20 $\mu\ell$ of PCR reaction containing 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 performed with 1 $\mu\ell$ cDNA template added to 10 $\mu\ell$ of 2X SYBR Premix Ex Taq (TaKaRa Bio, Inc.) containing specific primers at a concentration of 10 pM each. The reactions were carried out 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 2. The

Table 2. 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
Bax	F: 5'-TGCCTCAGGATGCATCTACC-3' R: 5'-AAGTAGAAAAGCGCGACCAC-3'	199	XM_003127290
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

fluorescence intensity was measured at the end of the extension phase of each cycle. The threshold value for the fluorescence intensity of all samples was set manually. The reaction cycle at which the PCR products exceeded this fluorescence intensity threshold was deemed the threshold cycle (Ct) in the exponential phase of the PCR amplification. The expression of each target gene was quantified relative to that of the internal control 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 FA treatment with various concentration (0, 5, 10, 20 μM) during IVM on intracellular levels of GSH and ROS was investigated. In experiment 2, the effect of FA treatment during IVM on subsequent embryonic development of PA was examined. In experiment 3, the effects of FA treatment on the mRNA expression levels (Bax, Nrf2, GCLC, GCLM, 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 FA on nuclear and cytoplasmic maturation during IVM

We performed porcine IVM supplemented with various concentration of FA (0, 5, 10, 20 μM). After 44 hours of maturation, there is no significant difference between control group and treatment group on maturation rates. We investigated the cytoplasmic maturation of porcine MII oocyte by examining intracellular GSH and ROS levels derived from the maturation medium supplemented with FA after IVM. The 5 μM of FA treatment group showed significantly increased ($P < 0.05$) intracellular GSH levels compared with the control group. The ROS generation levels significantly decreased at 20 μM of FA compared with the other groups in Figure. 1.

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

MII oocytes from each IVM groups were subjected to PA. The cleavages rate of PA was significantly increased ($P < 0.05$) treated with FA than control groups in Table 1. Embryonic developmental competence to the blastocyst stage and total cell numbers in blastocyst after PA with FA tended to increase compared with control groups. PA embryos treated

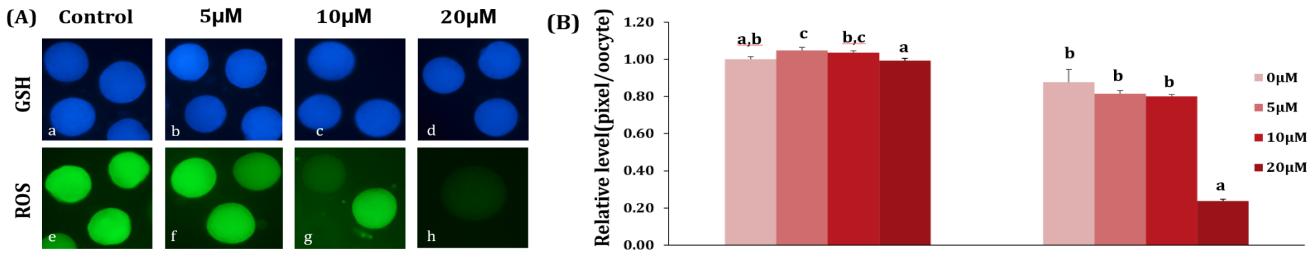

Figure 1. Epifluorescent photomicrographic images of in vitro–matured porcine oocytes. (A) Oocytes were stained with Cell Tracker Blue (a–d) and 20,70-dichlorodihydrofluorescein diacetate (e–h) to detect the intracellular levels of glutathione (GSH) and reactive oxygen species (ROS), respectively. Metaphase II oocytes derived from the maturation medium supplemented with 0 μM (a and e), 5 μM (b and f), 10 μM (c and g), and 20 μM of Ferulic Acid (FA, d and h). (B) The relative levels of intracellular GSH and ROS in in vitro–matured porcine oocytes among the four groups (0, 5, 10, and 20 μM of FA). Within each group end point (GSH and ROS), the bars with different letters (a–c) are significantly ($P < 0.05$) different. Total number of examined oocytes: GSH samples, N=30; ROS samples, N=30. The experiment was replicated three times.

with 10μM FA displayed the highest ($P < 0.05$) blastocyst formation rates (40.3%) and total cell numbers in blastocysts (51.9) compared with the other groups in Table 1.

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

To evaluate the effects of FA on the expression about antioxidative and anti-apoptotic related genes, we performed real-time PCR to anaylze the mRNA expression levels of *GCLC, GCLM, Keap1, Bcl-2, GSR, NRF2* and *NQO1* in the oocytes (Figure. 2) and the cumulus cells (Figure. 3) of control group and treated with 10μM of FA which is the most effective concentration at blastocyst formation and total cell numbers in blastocysts. *Keap1* gene expression is significantly decreased in both cumulus cells and oocytes treated with 10μM FA compared with control groups ($P < 0.05$). Also, *Nrf2* gene expression is significantly higher in oocytes and cumulus cells at treated groups ($P < 0.05$). There were no

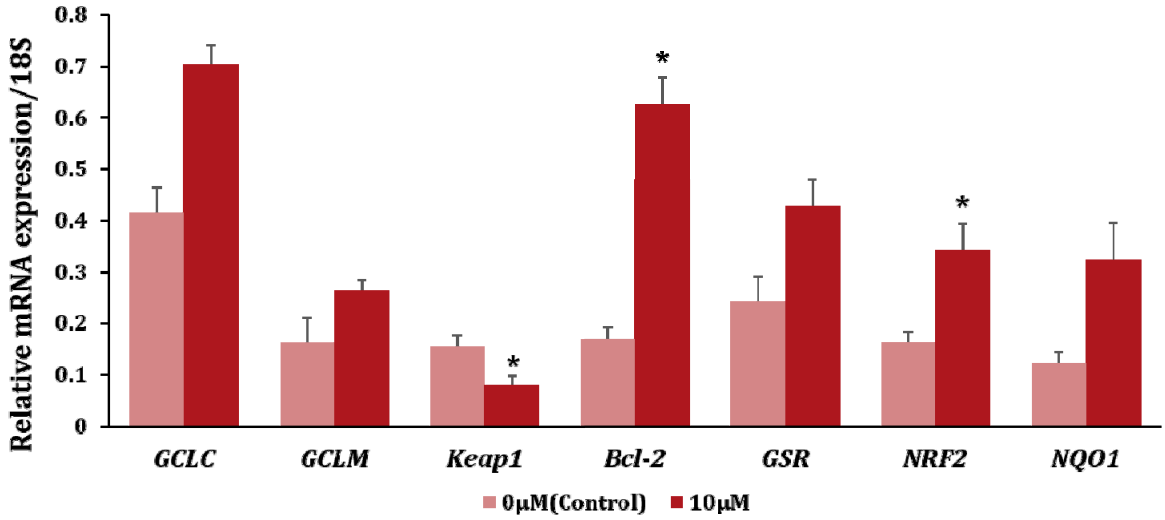

Figure 2. mRNA expression levels of *GCLC*, *GCLM*, *KEAP1*, *BCL2*, *GSR*, *NRF2* and *NQO1* in oocytes treated with FA during in vitro maturation (Mean ± SEM). The experiment was replicated three times.

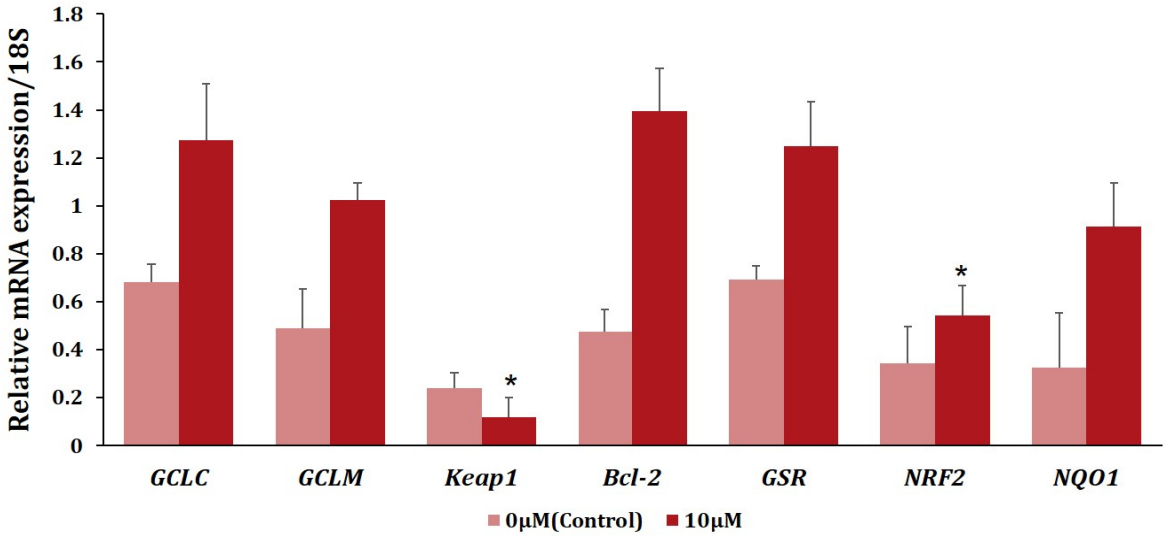

Figure 3. Messenger RNA expression levels of *GCLC*, *GCLM*, *KEAP1*, *BCL-2*, *GSR*, *Nrf2*, and *NQO1* relative to *18S* in cumulus cells treated with FA during *in vitro* maturation (Mean±SEM). The experiment was replicated three times.

significant differences in *GCLC, GCLM, Bcl-2, GSR,* and *NQO1* in oocytes and cumulus cells.

DISCUSSION

The purpose of this study was to examine the effects of FA on porcine IVM and subsequent embryonic developmental competence. Based on the experimental results, it can be seen that the antioxidant effect of FA occurs by controlling the *Keap1-Nrf2* pathway. The *Keap1-Nrf2* pathway regulates the major defense mechanisms of the cells against chemical / oxidative insults by regulating the expression of antioxidant enzymes, stress response enzymes, ABC transporters and various proteins (Kensler, et al. 2007). Under normal conditions, *Keap1* binds to *Nrf2* in the cytoplasm, acts as a negative repressor to *Nrf2* (Suzuki, et al. 2013). *Nrf2* is degraded by the proteasome. If *Nrf2* inducing chemicals such as toxic substances or oxidative insults are present, the action of *Keap1* is reduced and *Nrf2* is stabilized. Stabilized *Nrf2* migrates to the nucleus and activates numerous target genes (Baird and Dinkova-Kostova 2011). As a result of real-time PCR, FA supplementation downregulates *Keap1* expression and upregulates *Nrf2* expression. And measurement of intracellular GSH levels showed that the levels of intracellular GSH were increased compared to the control group when FA was treated. This suggests that upregulation of *Nrf2* induced intracellular GSH synthesis.

Increased intracellular GSH synthesis acts as a scavenger for ROS produced by excessive oxidative stress (Choe, et al. 2010). In addition, FA consists of phenolic nucleus and unsaturated side chain and it can exhibit antioxidant activity through its structural characteristics (Srinivasan, Sudheer and Menon 2007, Graf 1992). When radicals such as ROS are present, FA can combine with the hydrogen atoms of the radical to form resonance-stabilized phenoxy radicals which account for potent antioxidant activity (CASTELLUCCIO, et al. 1996). Further stabilization can be obtained through conjugation with the unsaturated side chain of FA. The intracellular ROS level was about 4 times lower than that of the control group (0.88 vs 0.24 respectively) when 20μM FA was treated, which is presumably due to excessive ROS scavenging. Physiological levels of ROS are necessary for cell development and growth (Truong, et al. 2016). If excessive ROS scavenging occurs, there is less ROS in the cells than in physiological demand, and cell development may be depressed. Therefore, we hypothesized that lower levels of intracellular GSH in the 20μM FA than in the control group are due to excessive ROS scavenging.

PA results showed that blastocyst formation rates and total cell numbers in blastocyst increased during FA treatment compared to control group. As a result of real time PCR, no significant increase of *Bcl-2* was observed in the treated group but in many previous studies, it can be confirmed that FA exhibits anti-apoptotic activity as *Bcl-2* independent manners (Khanduja, et al. 2006). Oxidative stress causes cellular DNA damage and is one of the factors causing apoptosis (Barzilai and Yamamoto 2004). Among them, ROS is the major substance causing oxidative stress-induced apoptosis. Therefore, it can be considered that the action of FA reduces cell damage by eliminating excessive ROS, resulting in anti-apoptotic activity.

In the natural environment, there are numerous oxidative stressors such as ultraviolet lights, excessive O_2, radiation, pollutants and toxicants (Franco, et al. 2009). When *IVM* techniques are used, they are basically exposed to excess oxygen (20% O_2) over the reproductive environment (5% O_2), resulting in oxidative damage to cells (Guerin, et al. 2001). Also various extrinsic factors can not be avoided and the cellular defense mechanisms are insufficient to prevent them. As a result, the efficiency of IVM is lower than in vivo system. Cell damage caused by ROS can alleviated by culturing COCs with anti-oxidants such as FA by its free radical scavenging capacity (Srinivasan, et al. 2006). Moreover, these data suggest that supplement during FA during porcine IVM improves blastocyst development of embryos.

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