A Dissertation for the Degree of Master of Science

# The Antioxidant Icariin Protects Porcine Oocytes against Damage during Aging *In Vitro*

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Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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# 항산화제 이카린이 체외에서 노화 동안 돼지 난자의 손상으로부터 보호에 미치는 영향

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**2020 년 2 월**

# The Antioxidant Icariin Protects Porcine Oocytes against Damage during Aging *In Vitro*

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(Supervised by Professor Se-Pill Park)

A thesis submitted in partial fulfillment of the requirement

For the degree of Master of Science

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This thesis has been examined and approved.

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# ABSTRACT

Icariin (ICA) is found in all species of *Epimedium* herbs, has strong antioxidant activity, and is thought to exert anti-aging effects *in vitro*. We investigated whether ICA treatment protects oocytes against aging *in vitro*. Treatment with 5 μM ICA (ICA-5) significantly decreased reactive oxygen species activity and increased the level of glutathione and mRNA expression of antioxidant genes (*SOD1, SOD2, PRDX5,* and *NFE2L2*) during aging. In addition, ICA-5 prevented defects in spindle formation and chromosomal alignment, and increased mRNA expression of cytoplasmic maturation factor genes (*BMP15, CCNB1, MOS,* and *GDF9*). ICA-5 prevented apoptosis, increased mRNA expression of anti-apoptotic genes (*BCL2L1* and *BIRC5*), and reduced mRNA expression of pro-apoptotic genes (*BAK1* and *CASP3*). Although the maturation and cleavage rates were similar in all groups, the total cell number per blastocyst and the percentage of apoptotic cells at the blastocyst stage were higher and lower, respectively, in the control and ICA-5 groups than in the aging group. These results indicate that ICA protects porcine oocytes against damage during aging *in vitro* by preventing oxidative stress.

Key words: *in vitro aging, porcine, oocyte, icariin, antioxidant*

# 1. INTRODUCTION

*In vitro* production technologies comprise three major consecutive steps: *in vitro* maturation (IVM) of immature oocytes, *in vitro* fertilization, and *in vitro* culture (IVC) of fertilized oocytes (Somfai & Hirao, 2017). The quality of *in vitro* matured oocytes determines the success of assisted reproductive technologies in mammalian species (Pawlak, Renska, Pers-Kamczyc, Warzych, & Lechniak, 2011; W. H. Wang, Abeydeera, Prather, & Day, 1998). In porcine *in vitro* production systems, immature oocytes are usually obtained from antral follicles measuring 2–8 mm in diameter of ovaries collected from a local slaughterhouse. Meiosis spontaneously resumes in immature mammalian oocytes following their removal from follicles and culture (Pincus & Enzmann, 1935). These oocytes undergo germinal vesicle breakdown after 16–20 hr and reach metaphase of the second meiotic division (MII) by 40 hr, and then meiosis arrests again until fertilization occurs (Motlik & Fulka, 1976; Wehrend & Meinecke, 2001). The nuclear and cytoplasmic events that occur during this process are collectively referred to as maturation, and are required for monospermic fertilization and early embryonic development (Wehrend & Meinecke, 2001). If fertilization does not occur within a specific period of time, the quality of unfertilized oocytes in the oviduct (*in vivo* aging) or culture (*in vitro* aging) will deteriorate over time (Y. L. Miao, Kikuchi, Sun, & Schatten, 2009). Aging oocytes often display abnormal spindle formation, disturbances in chromosome congression, mitochondrial alterations, and changes in gene and protein expression (Eichenlaub-Ritter, 2012). Consequently, many researchers have sought to develop methods that protect oocytes against aging *in vitro*.

Several studies reported that postovulatory aging is highly correlated with various oocyte defects, including precocious release of cortical granules, zona pellucida hardening, spindle and chromosomal abnormalities, a reduced fertilization ability, and abnormal development of

embryos and fetuses (Diaz & Esponda, 2004a, 2004b; Goud, Goud, Laverge, De Sutter, & Dhont, 1999; Saito et al., 1993; Tarin et al., 1999). In addition, postovulatory aging is accompanied by diverse molecular, cellular, and biochemical changes, such as dysfunction of mitochondria, generation of reactive oxygen species (ROS), reduced maturation-promoting factor activities, decreased expression of the anti-apoptotic factor B-cell lymphoma 2 (*BCL-2*), activation of caspase-3 (*CASP3*), and changes in epigenetic modifications (Y. L. Miao et al., 2009; H. Wang, Jo, Oh, & Kim, 2017). These aging-induced deleterious changes can reduce oocyte quality and thereby adversely affect fertilization and subsequent embryo development (Y. Miao et al., 2018).

The flavonoid icariin (ICA) is present in all species of *Epimedium* herbs and is extracted from the stem and leaves of the traditional Chinese medicinal plant *Epimedium brevicornum* Maxim (Herba Epimedii; family Berberidacae) (X. A. Li, Ho, Chen, & Hsiao, 2016; Ye et al., 2017). In general, the ICA content of *Epimedium* species varies from 0.003% to 1.55% based on reversed-phase high-performance liquid chromatography analysis, but can reach 3.69% in certain species (Wu, Lien, & Lien, 2003). ICA has a broad range of pharmacological and biological properties, including estrogenic activity, anti-inflammatory, antioxidant, and anti-tumor effects, and cardioprotective and neuroprotective properties (W. Li, Wang, Chu, Cui, & Bian, 2017; Liang, Vuorela, Vuorela, & Hiltunen, 1997; Liu et al., 2011; Makarova et al., 2007; T. Wang et al., 2007). ICA stimulates synthesis of nitric oxide synthase and increases nitric oxide production. These events correlate with activation of the PI3K/Akt and mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) kinase/ERK1/2 pathways (Chung et al., 2008).

This study investigated the antioxidant effects of various concentrations of ICA on porcine oocytes during aging *in vitro*. We analyzed the level of ROS, expression of antioxidant, maternal, and estrogen receptor genes, and spindle morphology in aging porcine

oocytes treated with or without ICA. In addition, we determined the developmental competence and quality of embryos derived through the parthenogenesis of these oocytes. Our results demonstrate that ICA protects porcine oocytes against damage during aging *in vitro* by preventing oxidative stress. These findings may be applicable to *in vitro* fertilization and help to protect oocytes against aging.

# 2. MATERIALS & METHODS

## 2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise.

## 2.2. Aging and IVM of porcine oocytes

Prepubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory in saline supplemented with 75 μg/mL penicillin G and 50 μg/mL streptomycin sulfate within 2 hr at 30–33°C. Cumulus-oocyte complexes (COCs) were aspirated from follicles with a diameter of 2–8 mm using an 18-gauge needle and a disposable 10 mL syringe. COCs were washed three times in tissue culture medium (TCM)-199–HEPES containing 0.1% (w/v) bovine serum albumin (BSA). Thereafter, COCs were matured in groups of 50 in 500 μL TCM-199 (Gibco, Grand Island, NY, USA) containing Earle's salts, 0.57 mM cysteine, 10 ng/mL epidermal growth factor, 0.5 μg/mL follicle-stimulating hormone, 0.5 μg/mL luteinizing hormone, and 10% (v/v) porcine follicular fluid under mineral oil for 44 hr at 38.8°C in 5% $CO_2$ in air. Oocyte aging was induced by culturing COCs for an additional 24 hr (total of 68 hr) in TCM-199.

## 2.3. Oocyte aging and ICA treatment

Mature oocytes were covered with mineral oil and cultured in a 4-well dish containing 500 μL TCM-199 at 38.8°C in a humidified atmosphere of 5% $CO_2$ in air. After maturation, MII oocytes were transferred to TCM-199 containing 0, 5, 50, and 500 μM ICA and cultured

for an additional 24 hr (total of 68 hr) as described above. After treatment, oocytes were collected and aging was assessed.

## 2.4. PA and embryo culture

Following maturation, cumulus cells were removed by pipetting in the presence of 1 mg/mL hyaluronidase for 2–3 min. PA was induced by treating oocytes with porcine zygote medium-5 containing 0.4% (w/v) BSA (IVC medium) and 5 μM $Ca^{2+}$ ionomycin for 5 min. After 3 hr of culture in IVC medium containing 7.5 μg/ml cytochalasin B, embryos were washed three times in the same medium and cultured for 7 days at 38.8°C in a humidified atmosphere of 5% $CO_2$ and 95% air. Oocytes and embryos were washed in Dulbecco's phosphate-buffered saline (DPBS) and either fixed in 3.7% (w/v) paraformaldehyde for 20 min and stored at 4°C, or snap-frozen in liquid nitrogen and stored at -70°C, depending on the experiment.

## 2.5. Measurement of intracellular ROS and GSH levels

DCFHDA and $CMF_2HC$ were used to determine the intracellular levels of ROS and GSH, respectively, as previously described (H. W. Yang et al., 1998; You, Kim, Lim, & Lee, 2010) with slight modifications. Briefly, cumulus cells were removed from COCs by pipetting in the presence of 0.1% (w/v) hyaluronidase. Denuded oocytes were incubated in DPBS containing 50 μM DCFHDA or 100 μM $CMF_2HC$ in the dark for 20 min at 38.8°C. Thereafter, oocytes were washed more than five times with DPBS containing 0.1% (w/v) BSA to completely remove excess dye and immediately analyzed by epifluorescence microscopy (Olympus, Tokyo, Japan). The ROS level was measured using excitation and

emission wavelengths of 450–490 nm and 515–565 nm, respectively. The excitation and emission wavelengths of $CMF_2HC$ are 371 and 464 nm, respectively. Grayscale images were acquired with a digital camera (Nikon, Tokyo, Japan) attached to the microscope, and mean grayscale values were calculated using ImageJ software (NIH, Bethesda, MD, USA). Background fluorescence values were subtracted from the final values before statistical analysis. The replicate was independently repeated 6–7 times, with 20–30 oocytes per experiment.

## 2.6. Immunofluorescence

Meiotic spindles and nuclei of oocytes were visualized after maturation. Cumulus cells were removed from porcine COCs matured for 44 hr (control) or an additional 24 hr (total of 68 hr) (0 and 5 μM ICA), and then oocytes were fixed overnight at 4°C in 4.0% (w/v) paraformaldehyde prepared in phosphate-buffered saline (PBS). Fixed oocytes were incubated for 30 min at 38.8°C with 0.5% (v/v) Triton X-100. After blocking for 1 hr with 1% BSA (w/v) prepared in PBS (blocking solution I), oocytes were incubated overnight at 4°C with a fluorescein isothiocyanate-conjugated anti-α-tubulin antibody (diluted 1:200 in blocking solution I). Nuclei were stained with Hoechst 33342 (1 μg/mL) for 30 min. Finally, oocytes were washed three times with PBS containing 0.1% (w/v) BSA, mounted on glass slides, and observed under an inverted Olympus IX-71 microscope. To further investigate the effect of ICA on spindle organization, spindles without abnormalities were classified as normal, whereas those in which chromosomes failed to align at the metaphase plate were classified as abnormal (Lenie, Cortvrindt, Eichenlaub-Ritter, & Smitz, 2008). Each experiment was independently repeated three times, and at least 20 oocytes were examined per group.

## 2.7. Terminal deoxynucleotidyl transferase dUTP nick-end labeling and Hoechst staining

At 7 days after PA, blastocysts were fixed overnight at 4°C with 4.0% (w/v) paraformaldehyde prepared in PBS, washed more than three times with PBS containing 0.1% BSA, and then incubated with 0.1% Triton X-100 at 38.8°C for 30 min. Blastocysts were incubated with fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase (In Situ Cell Death Detection Kit; Roche, Manheim, Germany) in the dark for 1 hr at 38.8°C. Mitotic and apoptotic cells were scored. Nuclei were stained with Hoechst 33342 (1 μg/mL) for 30 min, and embryos were washed with PBS containing 0.1% BSA. Blastocysts were mounted on glass slides and examined under an inverted Olympus IX-71 fluorescence microscope. The experiment was independently repeated 3–4 times, and at least 10–20 blastocysts were examined per group.

## 2.8. mRNA extraction and complementary DNA synthesis

mRNA was isolated from more than three biological replicates, with 30–40 oocytes per replicate, using a Dynabeads mRNA Direct Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. mRNA was collected in 10 μL elution buffer provided with the kit. Eluted RNA was reverse-transcribed into complementary DNA using an oligo (dT) 20 primer and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

## 2.9. Real-time RT-PCR

The protocol used was basically the same as that described previously (Lee, Sun, Choi,

Uhm, & Kim, 2012). Real-time RT-PCR was performed using the primer sets listed in Table 1 and a StepOnePlus Real-time PCR System (Applied Biosystems, Warrington, UK) with a final reaction volume of 20 μL containing SYBR Green PCR Master Mix (Applied Biosystems). The PCR conditions were as follows: 10 min at 95°C, followed by 39 cycles of 15 s at 95°C and 60 s at 54°C or 60°C. Samples were then cooled to 12°C. Relative gene expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) after normalization against the expression level of a housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*] or *β-actin*). The experiment was independently repeated five times.

**Table 1.** Primers used for real-time RT-PCR

| Gene | GenBank accession no. | Primer sequence* | Annealing temperature (°C) | Product size (bp) |
|---|---|---|---|---|
| *GAPDH* | AF017079.1 | F: GATGACATCAAGAAGGTGGT<br>R: CACTGTTAAAGTCAGAGGACAC | 54 | 100 |
| *GDF9* | XQ687750.1 | F: GTCTCCAACAAGAGAGAGATTC<br>R: CTGCCAGAAGAGTCATGTTAC | 54 | 109 |
| *BMP15* | NM_001005155.2 | F: GACACTGCCTTCTTGTTACTC<br>R: CTCTTGCCATAAACTCTTCC | 54 | 94 |
| *CCNB1* | NM_001170768.1 | F: ATACCTACTGGGTCGTGAAG<br>R: GGTCTCCTGTAGTAACCTGAAT | 54 | 97 |
| *MOS* | NM_001113219.1 | F: ACCTTACACCAGGTCATCTAC<br>R: GGAATACTTGAGACACTTCTCC | 54 | 105 |

| | | | | |
|---|---|---|---|---|
| *SOD1* | GU944822.1 | F: GTGTTAGTAACGGGAACCAT<br>R: GGATTCAGGATTGAAGTGAG | 54 | 120 |
| *SOD2* | NM_214127.2 | F: AGACCTGATTACCTGAAAGC<br>R: CTTGATGTACTCGGTGTGAG | 54 | 110 |
| *PRDX5* | AF110735.2 | F: GGCATGTCTGAGTGTTAATG<br>R: ATCTGTCTCCTTCCCAAAG | 54 | 118 |
| *NFE2L2* | Gu991000.1 | F: CTATGGAGACACACTGCTTG<br>R: ACAGGCTGTGTTTTAGGACT | 54 | 99 |
| *ESR1* | NM_214220.1 | F: TGGAGTGTACACGTTTCTGT<br>R: GTGTCTGTGATCTTGTCCAG | 54 | 87 |
| *ESR2* | NM_001001533.1 | F: AACTCTCCTGTCTCCTACAACT<br>R: GGCAGCTTTCTACATAGGAG | 54 | 91 |
| *BCL2L1* | NM_214285.1 | F: GGTTGACTTTCTCTCCTACAAG<br>R: CTCAGTTCTGTTCTCTTCCAC | 54 | 118 |
| *BIRC5* | NM_214141.1 | F: CTTCTGCTTCAAAGAGCTG<br>R: GGCTCTTTCTTTGTCCAGT | 54 | 154 |
| *FAS* | AJ001202.1 | F: GAGAGACAGAGGAAGACGAG<br>R: CTGTTCAGCTGTATCTTTGG | 54 | 194 |
| *BAK1* | AJ001204 | F: CTAGAACCTAGCAGCACCAT<br>R: CGATCTTGGTGAAGTACTC | 60 | 151 |
| *CASP3* | NM_214131 | F: GACTGCTGTAGAACTCTAACTGG<br>R: ATGTCATCTTCAGTCCCACT | 54 | 110 |

*F, forward; R, reverse.

## 2.10. Western blot analysis

The protocol was basically the same as that described previously (Lee et al., 2012). In brief, oocytes (40 per sample) were solubilized in 20 μL of 1× sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 50 μM dithiothreitol, and 0.01% (w/v) bromophenol blue or phenol red) and heated for 5 min at 95°C. Proteins were resolved on 5–12% Tris SDS-polyacrylamide gel electrophoresis gels for 1.5 hr at 80–100 V. Samples were then transferred to Hybond-ECL nitrocellulose membranes (Amersham, Buckinghamshire, UK) at 300 mA for 2 hr in transfer buffer (25 mM Tris, pH 8.5, containing 200 mM glycine and 20% [v/v] methanol). After blocking with 5% (w/v) nonfat milk prepared in PBS for 1 hr, the membranes were incubated for at least 2 hr with an anti-p44/42 MAPK or anti-phospho-p44/42 MAPK antibody diluted 1:500 in blocking solution (1× Tris-buffered saline, pH 7.5, containing 0.1% [v/v] Tween-20% and 5% [w/v] nonfat milk). Thereafter, the membranes were washed three times in TBST (20 mM Tris-HCl, pH 7.5, containing 250 mM NaCl and 0.1% [v/v] Tween-20) and incubated for 1 hr with anti-rabbit IgG-horseradish peroxidase diluted 1:2,000 in blocking solution. After three washes with TBST, immunoreactive protein bands were visualized to X-ray films using chemiluminescent luminol reagent (Invitrogen) in the dark room. The experiment was independently repeated three times.

## 2.11. Statistical analysis

The general linear model procedure within the Statistical Analysis System (SAS User's Guide, 1985, Statistical Analysis System Inc., Cary, NC) was used to analyze data from all experiments. The paired Student's t test was used to compare relative gene expression. $p < 0.05$ was considered significant.

# 3. RESULTS

## 3.1. ICA enhances the *in vitro* embryo development of aging porcine oocytes

To determine the optimal concentration of ICA, porcine oocytes were matured for 44 hr (control) or for an additional 24 hr (total of 68 hr) in the presence of 0, 5, 50, and 500 μM ICA (aging, ICA-5, ICA-50, and ICA-500, respectively). The percentage of oocytes that reached MII did not differ between the groups. Following parthenogenetic activation (PA), the percentage of oocytes that underwent cleavage and reached the 2–4-cell stage did not differ between the aging, ICA-5, ICA-50, ICA-500, and control groups (control, 72.8 ± 5.6%; aging, 80.2 ± 5.0%; ICA-5, 86.0 ± 4.7%; ICA-50, 77.7 ± 3.7%; and ICA-500, 81.6 ± 4.1%; Table 1). The percentage of cleaved oocytes that reached the blastocyst stage on Day 7 was significantly higher ($p < 0.05$) in the control and ICA-treatde groups than in the aging group, and was similar in the ICA-5, ICA-50, and ICA-500 groups (control, 45.8 ± 3.2%; aging, 22.9 ± 1.9%; ICA-5, 34.8 ± 2.3%; ICA-50, 36.8 ± 4.8%; and ICA-500, 37.8 ± 2.3%; Table 1). Therefore, the control, aging, and ICA-5 groups were compared in subsequent experiments. The replicate was independently repeated 7 times, with 50–60 oocytes per experiment. All data are presented as the means ± SEM.

**Table 2.** Effect of ICA treatment during aging of porcine oocytes in vitro on subsequent embryo development

| Treatment group | ICA concentration | No. of germinal | No. (%) of Surviving | No. (%) of cleaved | No. (%) of Blastocysts |
|---|---|---|---|---|---|

| | (μM) | vesicle oocytes | Oocytes[1] | oocytes on Day 2[2] | on Day 7[3] |
|---|---|---|---|---|---|
| Control | 0 | 56 | 51 (91.4 ± 1.6) | 37 (72.8 ± 5.6) | 17 (45.8 ± 3.2)$^{c}$ |
| Aging | 0 | 56 | 51 (89.8 ± 1.6) | 41 (80.2 ± 5.0) | 9 (22.9 ± 1.9)$^{a}$ |
| ICA-5 | 5 | 56 | 52 (92.0 ± 0.8) | 45 (86.0 ± 4.7) | 16 (34.8 ± 2.3)$^{bc}$ |
| ICA-50 | 50 | 59 | 54 (91.5 ± 2.9) | 42 (77.7 ± 3.7) | 15 (36.8 ± 4.8)$^{b}$ |
| ICA-500 | 500 | 59 | 53 (90.5 ± 1.7) | 43 (81.6 ± 4.1) | 16 (37.8 ± 2.3)$^{b}$ |

[1]The percentage of oocytes that reached MII. [2]The percentage of oocytes that underwent cleavage. [3]The percentage of cleaved oocytes that reached the blastocyst stage on Day 7. Values are means ± SEM of independent experiments. Values with different superscript letters are significantly different (a-cp < 0.05). ICA, icariin.

### 3.2. ICA reduces the level of ROS in aging porcine oocytes *in vitro*

The effects of ICA on the levels of ROS and glutathione (GSH) were analyzed by staining oocytes with dichlorohydrofluorescein diacetate (DCFHDA) and CellTracker™ Blue 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin ($CMF_2HC$), respectively (Figure 1A). The staining intensity of ROS was significantly lower ($p < 0.05$) in the ICA-5 group than in the aging group, but did not differ between the control and aging groups (control, $1.0 \pm 0.0$ pixels/oocyte; aging, $1.0 \pm 0.0$ pixels/oocyte; and ICA-5, $0.8 \pm 0.1$ pixels/oocyte; Figure 1B). The staining intensity of GSH was significantly higher ($p < 0.05$) in the control and ICA-5 groups than in the aging group (control, $1.0 \pm 0.0$ pixels/oocyte; aging, $0.8 \pm 0.1$ pixels/oocyte; and ICA-5, $1.1 \pm 0.1$ pixels/oocyte; Figure 1C).

Expression of the antioxidant genes superoxide dismutase 1 (*SOD1*), superoxide dismutase 2 (*SOD2*), peroxiredoxin 5 (*PRDX5*), and nuclear factor erythroid 2-like 2 (*NFE2L2*) was analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR; Figure 1D). mRNA expression of *SOD1* and *PRDX5* was significantly higher ($p < 0.05$) in the ICA-5 group than in the aging group, and was similar in the control and aging groups. mRNA expression of *SOD2* was significantly higher ($p < 0.05$) in the ICA-5 group than in the aging group, but similar to control. mRNA expression of *NFE2L2* was significantly higher ($p < 0.05$) in the ICA-5 group than in the aging and control groups. All data are presented as the means ± SEM.

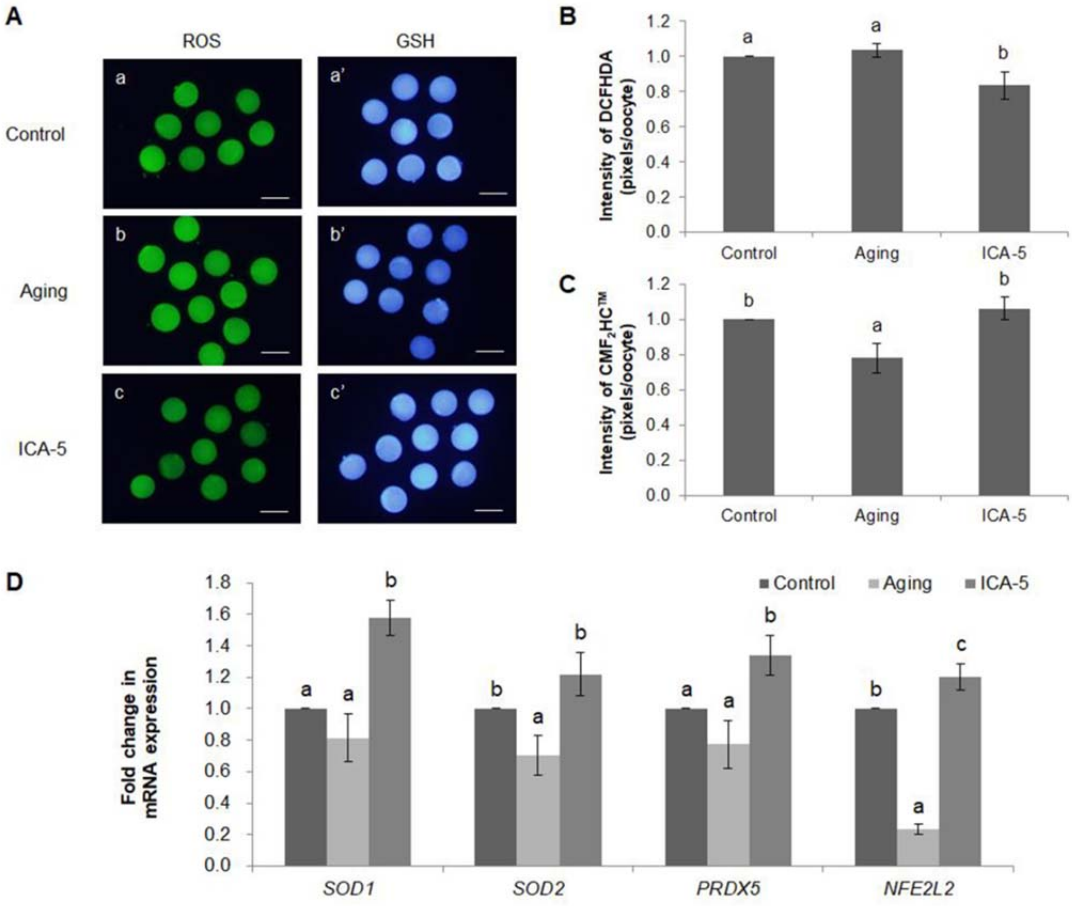


**Fig. 1. Antioxidant effect of ICA during aging of porcine oocytes *in vitro*.** A: Epifluorescence images of oocytes stained with DCHFDA (green) and CellTracker™ Blue $CMF_2HC$ (blue). a and a': control group; b and b': aging group; and c and c': ICA-5 group. a, b, and c: ROS staining; a', b', and c': GSH staining. B: Fluorescence intensities of intracellular ROS staining. C: Fluorescence intensities of intracellular GSH staining. D: Relative expression of the antioxidant genes *SOD1, SOD2, PRDX5,* and *NFE2L2*. Data were derived from 3–4 independent replicates per group. Data are the means ± SEM ($^{a-c}p < 0.05$). Scale bar = 120 μm.

### 3.3. ICA prevents aberrant spindle organization and chromosome misalignment in aging porcine oocytes *in vitro*

The percentage of oocytes with normal meiotic spindles was significantly higher ($p < 0.05$) in the ICA-5 group than in the aging group, and was similar in the control and ICA-5 groups (control, 80.3 ± 0.8%; aging, 72.6 ± 2.1%; and ICA-5, 86.7 ± 4.5%; Figure 2). All data are presented as the means ± SEM.

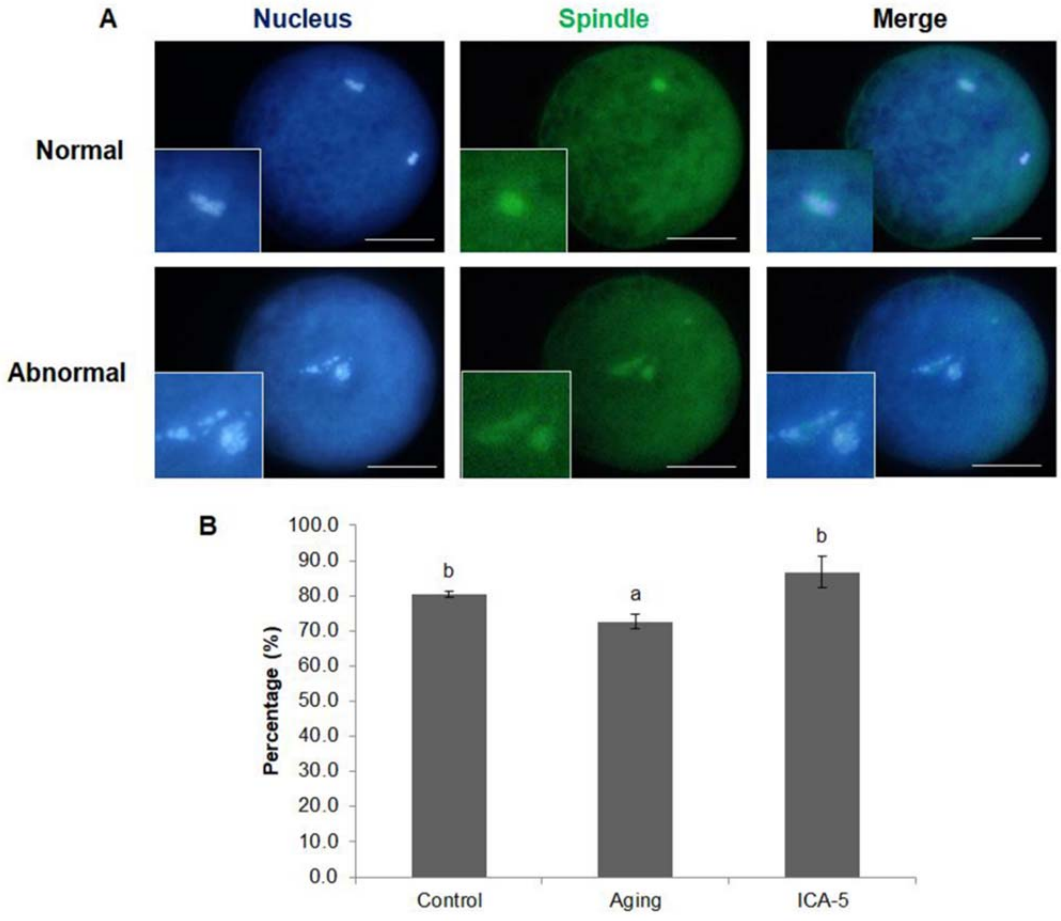


**Fig. 2. Effect of ICA on meiotic spindle morphology in porcine oocytes during aging *in vitro*.** A: Normal and abnormal chromosome alignment and meiotic spindle formation in oocytes. B: Percentage of oocytes in which the morphologies of chromosomes and the

meiotic spindle were normal. Data were derived from three independent replicates per group. Data are the means ± SEM ($^{a-b}p < 0.05$). Scale bar = 50 μm.

### 3.4. ICA increases expression of cytoplasmic maturation markers in aging porcine oocytes *in vitro*

To investigate the effect of ICA on cytoplasmic maturation of aging oocytes, we examined maternal gene expression and MAPK activity (Figure 3). Expression of the cytoplasmic maturation marker genes bone morphogenetic protein 15 (*BMP15*), cyclin B1 (*CCNB1*), MOS proto-oncogene, serine/threonine kinase (*MOS*), and growth differentiation factor-9 (*GDF9*) was analyzed by real-time RT-PCR (Figure 3A). mRNA expression of *CCNB1, MOS,* and *GDF9* was significantly higher ($p < 0.05$) and lower ($p < 0.05$) in the ICA-5 group than in the aging and control groups, respectively. mRNA expression of *BMP15* was significantly higher ($p < 0.05$) in the ICA-5 group than in the aging and control groups. Western blotting revealed that phosphorylated p44/42 MAPK (phospho-p44/42 MAPK), the active form of this kinase, migrated as a doublet in lysates of matured and aged porcine oocytes. The relative ratio of phospho-p44/42 MAPK to total p44/42 MAPK did not significantly differ ($p < 0.05$) between the groups (control, $1.0 \pm 0.0$; aging, $0.9 \pm 0.1$; and ICA-5, $1.0 \pm 0.1$; Figure 3B). All data are presented as the means ± SEM.

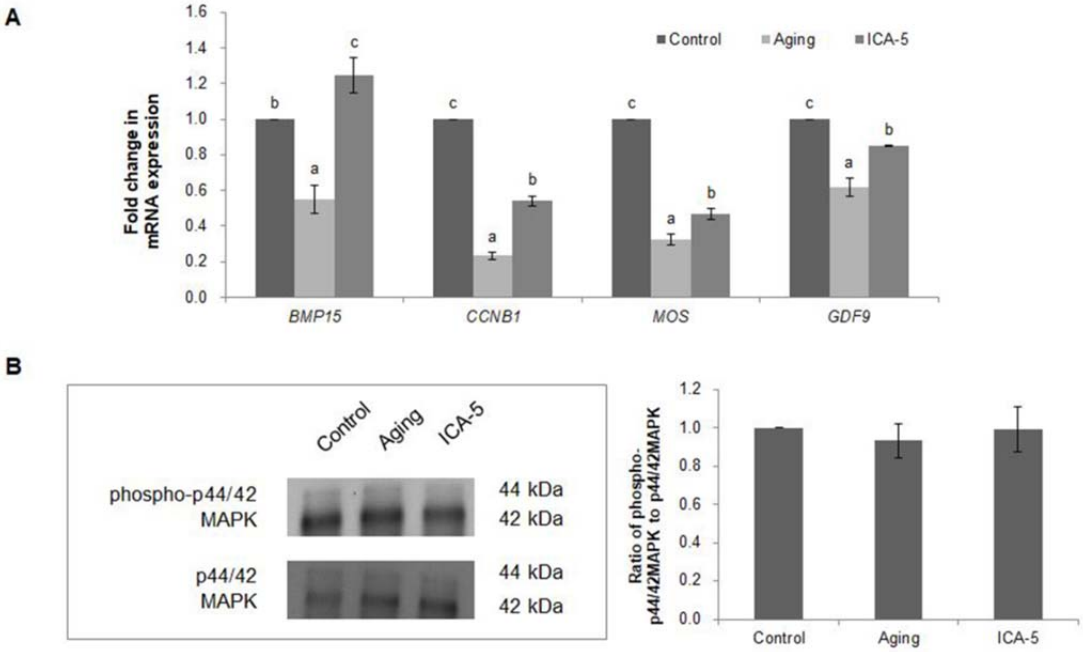


**Fig. 3. Effect of ICA treatment during aging of porcine oocytes *in vitro* on expression of maternal genes and MAPK activity.** A: Maternal gene expression. B: MAPK activity. Data were normalized against the levels in the control group and were derived from three or five independent replicates per group. Data are the means ± SEM ($^{a-c}p < 0.05$).

### 3.5. ICA increases expression of estrogen receptor genes in aging porcine oocytes *in vitro*

The effects of ICA on mRNA expression of estrogen receptor 1 (*ESR1*) and 2 (*ESR2*) were analyzed by real-time RT-PCR to determine if ICA activates these receptors. mRNA expression of *ESR1* was significantly higher ($p < 0.05$) and lower ($p < 0.05$) in the ICA-5 group than in the aging and control groups, respectively (Figure 4). mRNA expression of *ESR2* was significantly higher ($p < 0.05$) in the ICA-5 group than in the control and aging groups (Figure 4). All data are presented as the means ± SEM.

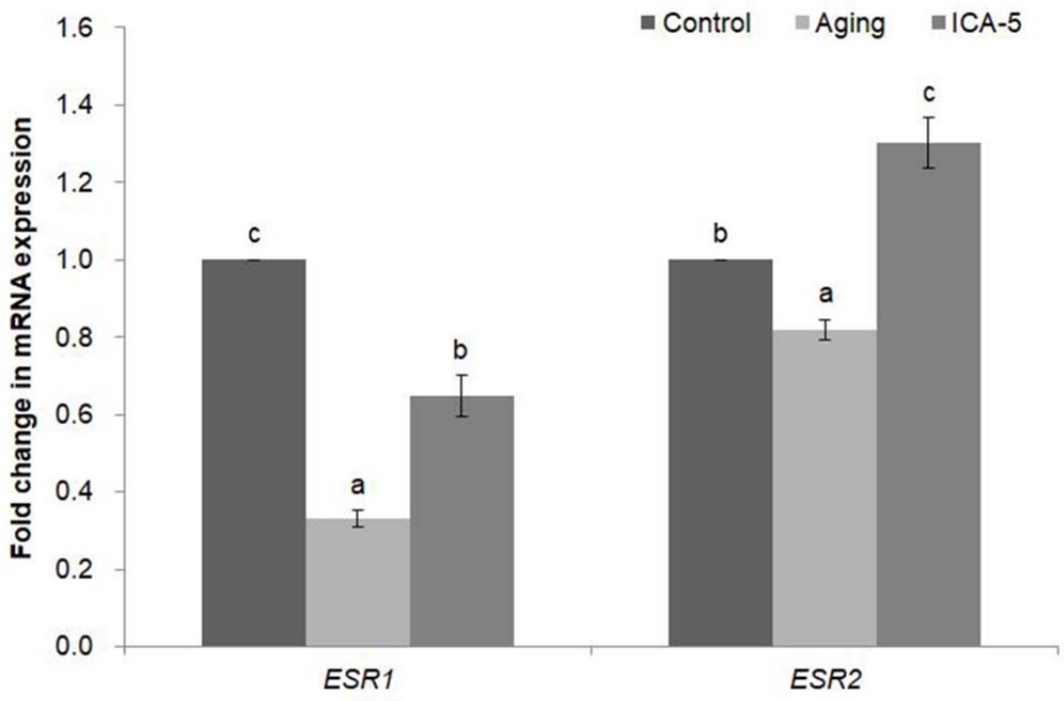

**Fig. 4. Effect of ICA treatment during aging of porcine oocytes in vitro on expression of estrogen receptor genes.** Data were derived from five independent replicates per group. Data are the means ± SEM ($^{a-c}p < 0.05$).

### 3.6. ICA changes expression of apoptosis-related genes in aging porcine oocytes *in vitro*

Expression of the apoptosis-related genes BCL2-like 1 (*BCL2L1*), baculoviral IAP repeat-containing 5 (*BIRC5*), Fas cell surface death receptor (*FAS*), BCL2 antagonist/killer 1 (*BAK1*), and *CASP3* was analyzed by real-time RT-PCR (Figure 5). mRNA expression of *BCL2L1* was significantly higher ($p < 0.05$) in the ICA-5 group than in the control and aging groups. mRNA expression of *BIRC5* was significantly higher ($p < 0.05$) in the ICA-5 group than in the aging group, and was similar in the control and ICA-5 groups. mRNA expression of *FAS* was significantly higher ($p < 0.05$) in the aging group than in the control group, was similar in the control and ICA-5 groups, and did not significantly differ between the aging and ICA-5 groups. mRNA expression of *BAK1* and *CASP3* was significantly higher ($p < 0.05$) and lower ($p < 0.05$) in the ICA-5 group than in the control and aging groups, respectively. All data are presented as the means ± SEM.

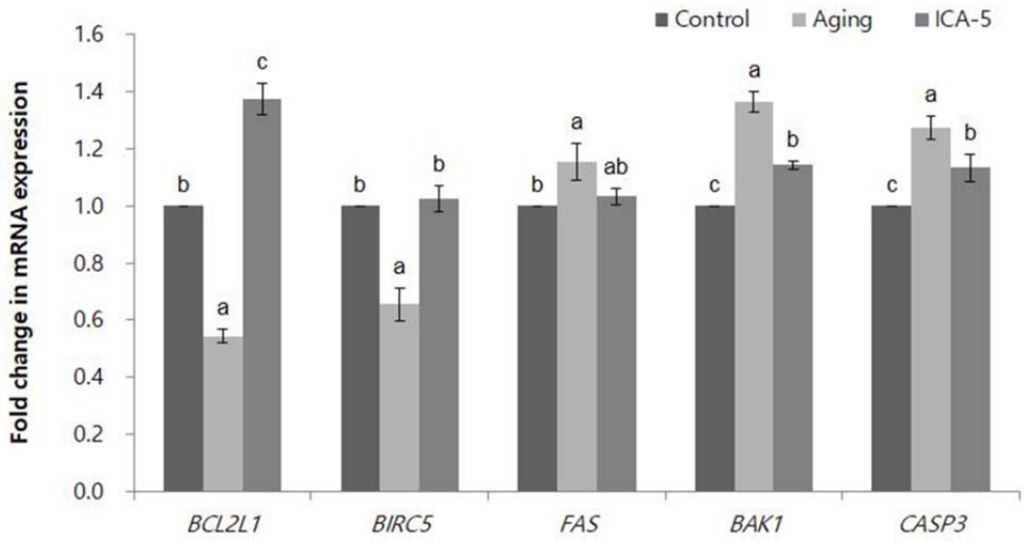

**Fig. 5. Effect of ICA treatment during aging of porcine oocytes *in vitro* on expression of apoptosis-related genes.** Data were derived from five independent replicates per group. Data are the means ± SEM ($^{a–c}p < 0.05$).

### 3.7. ICA improves the developmental capacity and quality of embryos derived from aging porcine oocytes *in vitro*

To investigate whether ICA treatment during IVM of oocytes influences subsequent embryo development and quality, oocytes were matured in the presence or absence of 5 μM ICA and then parthenogenetically activated. The total number of cells per blastocyst was significantly lower ($p < 0.05$) in the ICA-5 group than in the control group. However, the aging group was significantly lower ($p < 0.05$) than the ICA-5 group (control, $90.4 \pm 1.4$; aging, $60.9 \pm 3.3$; and ICA-5, $73.8 \pm 4.2$; Figure 6B). Genomic DNA fragmentation was assessed by terminal deoxynucleotidyl transferase dUTP nick-end labeling to detect apoptotic cells in blastocysts. The percentage of apoptotic cells was significantly higher ($p < 0.05$) in the aging group than in the control and ICA-5 groups (control, $1.8 \pm 0.6\%$; aging, $6.0 \pm 1.6\%$; and ICA-5, $2.3 \pm 0.7\%$; Figure 6C). All data are presented as the means ± SEM.

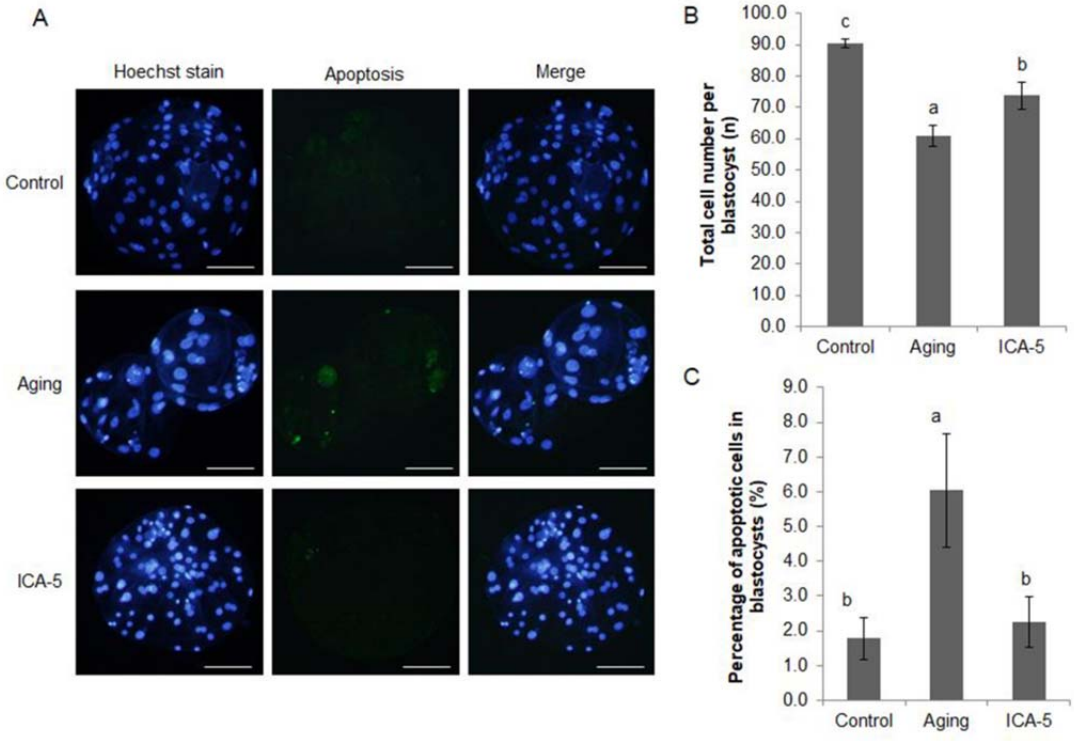


**Fig. 6. Effect of ICA treatment during aging of porcine oocytes *in vitro* on subsequent embryo quality.** A: Blastocyst staining. B: Total cell number per blastocyst. C: Percentage of apoptotic cells in blastocysts. Data were derived from 3–4 independent replicates per group. Data are the means ± SEM ($^{a–c}p < 0.05$). Scale bar = 50 μm.

# 4. DISSCUSTION

The mechanism by which oocytes are protected from aging *in vitro* is unknown. This study investigated the effects of the antioxidant ICA on aging of porcine oocytes *in vitro*. Some studies have conducted parthenogenesis instead of IVF or intracytoplasmic sperm injection (ICSI). Before implantation, parthenogenesis can show the outcome of IVF or ICSI roughly. It is not exactly the same, but you can see the approximate pattern. Treatment with 5 μM ICA during 24 hr of aging significantly decreased than the aging group the level of ROS and expression of pro-apoptotic genes (*BAK1* and *CASP3*), and significantly increased expression of antioxidant genes (*SOD1, SOD2, PRDX5,* and *NFE2L2*), expression of maternal genes (*BMP15, CCNB1, MOS,* and *GDF9*), expression of anti-apoptotic genes (*BCL2L1* and *BIRC5*), the level of GSH, and the percentage of oocytes with normal spindles. In addition, embryos derived from these ICA-treated oocytes exhibited an improved developmental capacity and quality. This study demonstrates that ICA protects oocytes against damage during aging *in vitro*.

Others have shown that oxidative stress arises in postovulatory aging oocytes, in which the level of ROS is increased and antioxidant protection is concomitantly decreased (Dai et al., 2017; Kim et al., 2019; Y. Miao et al., 2018). Although intracellular GSH plays an important role in protecting oocytes against oxidative damage, its level gradually decreases during aging (Boerjan & de Boer, 1990). The level of ROS was significantly lower in the ICA-5 group than in the control and aging groups, but was similar in the control and aging groups (Figure 1B). Moreover, the level of GSH was significantly higher in the ICA-5 and control groups than in the aging group (Figure 1C). These results suggest that ICA decreases the level of ROS and prevents decrease in GSH level during *in vitro* aging of oocytes. In

addition, in addition, previous studies have shown that icariin inhibits ROS production in lipopolysaccharide-treated microglia (Zeng, Fu, Liu, & Wang, 2010). Expression of antioxidant genes (*SOD1, SOD2, PRDX5,* and *NFE2L2*) was higher in the ICA-5 group than in the aging group, expression of *SOD1* and *PRDX5* was similar in the control and aging groups, expression of *SOD2* was similar in the control and ICA-5 groups, and expression of *NFE2L2* was higher in the ICA-5 group than in the control group (Figure 1D). *SOD* is an antioxidant enzyme that plays an important role in the defense system against ROS and is an important ROS scavenger. *SOD1* converts two superoxide anions, which are normal products of cellular respiration, into hydrogen peroxide and oxygen [$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$] (McCord & Fridovich, 1969). *SOD2* reduces the superoxide anion produced as a byproduct of oxidative phosphorylation to generate hydrogen peroxide and oxygen (Y. Li et al., 1995; Margalit et al., 2015). *PRDX5* protects cells against ROS by prioritizing the elimination of hydrogen peroxide and alkyl hydroperoxides (Dubuisson et al., 2004; Knoops, Goemaere, Van der Eecken, & Declercq, 2011; Rhee, Woo, Kil, & Bae, 2012). Other studies reported that *PRDX5* is only found in specific cellular organelles, namely, peroxisomes and mitochondria, and plays a role in protecting them against neurological damage caused by excessive ROS (Banmeyer et al., 2004; Kropotov, Usmanova, Serikov, Zhivotovsky, & Tomilin, 2007; Park et al., 2016; Sun et al., 2010). *NFE2L2* transactivates genes containing antioxidant response elements and coordinates expression of cytoprotective genes to protect cells against oxidative stress (Itoh, Ye, Matsumiya, Tanji, & Ozaki, 2015). These results suggest that the expression of antioxidant genes was decreased in the aging group and that ICA protects oocyte against ROS by preventing reduction of the expression of antioxidant genes as shown in ICA-5 group.

Chromosome condensation is the most noticeable event in meiotic maturation and is important for the formation and proper separation of chromosomes (Jelinkova & Kubelka,

2006). Postovulatory oocyte aging changes the levels of spindle-associated proteins and induces acetylation of α-tubulin (Cecconi et al., 2014). The percentage of oocytes with normal spindles was lower in the aging group than in the control group, but was similar in the ICA-5 and control groups (Figure 2B). Our results demonstrate that treatment with ICA during aging of oocytes *in vitro* protects chromosomes and spindles in MII.

Expression of the maternal genes *BMP15, CCNB1, MOS,* and *GDF9* was higher in the ICA-5 group than in the aging group, expression of *BMP15* was higher in the ICA-5 group than in the control group, and expression of *CCNB1, MOS,* and *GDF9* was lower in the ICA-5 group than in the control group (Figure 3A). *BMP15* prevents apoptosis of cumulus cells (Hussein, Froiland, Amato, Thompson, & Gilchrist, 2005). *CCNB1* forms a complex with cyclin-dependent kinase 1 to allow cell cycle transition from G2 to M phase (Robert, Hue, McGraw, Gagne, & Sirard, 2002; Zhang et al., 2011). *MOS* plays a vital role in oocyte maturation by participating in MII arrest and asymmetric meiotic division (Evangelou et al., 2002). *GDF9* has multifunctional roles in communication of oocyte granulosa cells and regulation of the differentiation and function of follicular cells (Elvin, Clark, Wang, Wolfman, & Matzuk, 1999). *BMP15* and *GDF9* interact and thereby play vital roles in follicular development, atresia, ovulation, formation of fertilized eggs, and maintenance of reproduction (Glister, Kemp, & Knight, 2004; Su et al., 2004; Yan et al., 2001; Yoon et al., 2015). These results suggest that the expression of maternal genes was decreased in the aging group and that ICA prevents deterioration of oocyte quality by preventing reduction of the expression of maternal genes as shown in ICA-5 group.

In the present study, we determined the expression levels of *ESR1* and *ESR2* to investigate whether ICA enters oocytes via estrogen receptors. Expression of both genes was significantly higher in the ICA-5 group than in the aging group, expression of *ESR1* was

lower in the ICA-5 group than in the control group, and expression of *ESR2* was higher in the ICA-5 group than in the control group (Figure 4). These results suggest that the expression of receptor genes was decreased in the aging group and that ICA prevents decrease of receptor genes against *in vitro* aging of oocytes as shown in ICA-5 group. In addition, ICA might act via estrogen receptors or in cooperation with estrogen receptor signaling (Song, Zhao, Zhang, Li, & Zhou, 2013). The two types of estrogen receptor are ERα and ERβ, which are encoded by *ESR1* and *ESR2*, respectively (Kim et al., 2019). ERα and ERβ antagonize the actions of one another in many tissues (Khalid & Krum, 2016). Variation in pre-mRNA splicing and expression of *ESR1* alters the function of ERα (Ghali et al., 2018). ERβ is homologous to ERα, and these two proteins have similar, but not identical, tissue distributions (Mosselman, Polman, & Dijkema, 1996). Our results demonstrate that ICA enters porcine oocytes through estrogen receptors.

Expression of the anti-apoptotic genes *BCL2L1* and *BIRC5* was higher in the ICA-5 group than in the aging group (Figure 5). Expression of the pro-apoptotic gene *FAS* was similar in the ICA-5 and aging groups, while expression of the pro-apoptotic gene *BAK1* was lower in the ICA-5 group than in the aging group (Figure 5). Apoptosis occurs during development and aging, and is also a homeostatic mechanism to maintain cell populations in tissues (Elmore, 2007). In addition, apoptosis functions as a defense mechanism when cells are damaged by disease or noxious agents or in immune reactions (Norbury & Hickson, 2001). Apoptosis is a form of programmed cell death that kills individual cells in an organism while preserving the overall structure of the surrounding tissue (Parrish, Freel, & Kornbluth, 2013). Excessive apoptosis can affect blastocyst maturation, induce death of an early embryo, and cause fetal deformities (Brison & Schultz, 1997). Several genes control apoptosis. As a dominant inhibitor of apoptosis, *BCL2L1* is a central regulator of programmed cell death and an important anti-cancer drug target (Oltersdorf et al., 2005). *BIRC5* is a member of the

inhibitors of apoptosis family and is involved in regulation of cell division and inhibition of apoptosis (Contis, Lykoudis, Goula, Karandrea, & Kondi-Pafiti, 2018). *FAS*-mediated apoptosis is involved in development of immunity, regulation of immune responses, and killing of viral-infected cells and tumor targets (Eischen & Leibson, 1997; Krammer et al., 1994; Nagata & Golstein, 1995). *BAK1* is a member of the *BCL-2* family and is an important regulator of mitochondrial apoptosis (Birkinshaw & Czabotar, 2017). Caspases are the primary drivers of apoptotic cell death and cleave cellular proteins, which is critical for dismantling dying cells (Parrish et al., 2013). *CASP3* is the most well-characterized effector caspase (Parrish et al., 2013). Specifically, *CASP3* is the executioner caspase and functions in the final phase of apoptosis. Consequently, it is cleaved and activated during late apoptotic events (Riedl & Shi, 2004). These results suggest that the expression of anti-apoptotic genes was decreased in the aging group and that ICA prevents decrease of anti-apoptotic genes against *in vitro* aging of oocytes as shown in ICA-5 group. In addition, the expression of pro-apoptotic genes was increased in the aging group and ICA prevents the expression of pro-apoptotic genes from increasing as shown in ICA-5 group. Thus, these results suggest that ICA prevents apoptosis induced by oxidative stress. Other studies reported that ICA prevented apoptosis in human vascular endothelial cells following oxidized low‑density lipoprotein treatment, in via the regulation of protein and mRNA expression levels of *BCL-2* and *CASP3* (Hu et al., 2018).

The easiest way to assess the quality of oocytes *in vitro* is to calculate the developmental rate. This rate determines the efficiency with which embryos are produced *in vitro*. Aging negatively affects oocyte competency and embryo development (Marshall & Rivera, 2018), and reduces the cleavage rate of oocytes (W. J. Yang et al., 2015). The percentage of oocytes that underwent cleavage did not significantly differ between the control, aging, and ICA-5 groups (Table 2). The total number of cells per blastocyst and the percentage of apoptotic

cells in blastocysts were determined to investigate blastocyst quality (Figure 6). The total cell number per blastocyst was higher in the ICA-5 group than in the aging group, and was higher in the control group than in the ICA-5 group (Figure 6B). The percentage of apoptotic cells in blastocysts was significantly lower in the ICA-5 group than in the aging group, and did not significantly differ between the control and ICA-5 groups (Figure 6C). Thus, the developmental rate was decreased in the aging group and this was prevented in the ICA-5 group, suggesting that treatment with 5 μM ICA protects oocytes against aging *in vitro*.

In conclusion, this study indicates that treatment with 5 μM ICA reduces the level of ROS, prevents decrease of the expression of antioxidant gene, effectively protects oocytes against oxidative stress, prevents decrease of the expression of maternal gene, and thereby minimizes the deterioration in oocyte quality during aging *in vitro*. Moreover, prevents decrease of the expression of anti-apoptotic gene, prevents increase of the expression of pro-apoptotic gene, enhancing blastocyst formation and development, thereby increasing production of good-quality blastocysts. Therefore, ICA can be used to improve assisted reproductive technologies.

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# ABSTRACT IN KOREAN

이카린 (ICA)은 모든 종의 에피메디움 허브에서 존재하며 강력한 항산화 활성을 가지며 시험관 내에서 노화 방지 효과를 발휘하는 것으로 생각된다. 우리는 ICA 처리가 시험관 내 노화에 대해 난모세포를 보호하는지 여부를 조사하였다. 5 μM ICA (ICA-5)로 처리하면 노화 동안 활성산소 수준은 크게 감소하고 글루타티온 수준과 항산화 유전자 (*SOD1, SOD2, PRDX5* 및 *NFE2L2*)의 mRNA 발현이 증가하였다. 또한, ICA-5 는 스핀들 형성 및 염색체 정렬에서의 결함을 방지하고 세포질 성숙 인자 유전자 (*BMP15, CCNB1, MOS* 및 *GDF9*)의 mRNA 발현을 증가시켰다. ICA-5 는 세포사멸을 방지하고, 항-세포사멸 유전자 (*BCL2L1* 및 *BIRC5*)의 mRNA 발현을 증가시키고, 세포사멸 유전자 (*BAK1* 및 *CASP3*)의 mRNA 발현을 감소시켰다. 성숙 및 난할율은 모든 그룹에서 유사하지만, 배반포당 총 세포 수 및 배반포 단계에서의 세포사멸 세포의 백분율은 각각 노화 그룹보다 대조군 및 ICA-5 그룹에서 더 높고 더 낮았다. 이러한 결과는 ICA 가 산화스트레스를 방지함으로써 시험관내 노화 동안 돼지의 난모세포를 손상으로부터 보호한다는 것을 나타낸다.

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