Thesis for the degree of Master of Science

Ellagic Acid durng *In Vitro* Maturation of Porcine Oocytes Improves Development Capacity after Parthenotes and Somatic Cell Nuclear Transfer

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Ellagic Acid during *In Vitro* Maturation of Porcine Oocytes Improves Development Capacity after Parthenotes and Somatic Cell Nuclear Transfer

A Thesis submitted to the graduate school of
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The thesis for the degree of Master of Science
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has been approved by the dissertation committee.

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Ellagic acid (EA) is a natural polyphenol and a free radical scavenger with antioxidant properties. This study investigated the protective effects of EA during *in vitro* maturation (IVM) of porcine oocytes. To determine the optimal concentration, IVM medium was supplemented with various concentrations of EA. Treatment with 10 μM EA (10 EA) resulted in the highest cleavage rate, blastocyst formation rate, and total cell number per blastocyst and the lowest percentage of apoptotic cell in parthenogenetic blastocysts. In the 10 EA group, abnormal spindle and chromosome misalignment were rescued and the ratio of phosphorylated p44/42 to total p44/42 was increased. Furthermore, the reactive oxygen species and glutathione levels were significantly decreased and increased, respectively, and antioxidant genes (*Nrf2, HO-1, CAT,* and *SOD1*) were significantly upregulated in the 10 EA group. mRNA expression of developmental-related (*CDX2*, *POU5F1*, and *SOX2*) and anti-apoptotic (*BCL2L1*) genes was significantly upregulated in the 10 EA group, while mRNA expression of pro-apoptotic genes (*BAK*, *FAS*, and *CASP3*) was significantly

downregulated. Ultimately, following somatic cell nuclear transfer, blastocyst formation rate was significantly increased and the percentage of apoptotic cell in blastocysts was significantly decreased in the 10 EA group. In conclusion, addition of 10 EA to IVM medium improved oocyte maturation and subsequent embryo development capacity through antioxidant mechanisms. These findings suggest that EA can enhance the efficiencies of assisted reproductive technologies.

Key words: *Ellagic acid, Antioxidant, In vitro mturation, Porcine oocytes, Reactive oxygen spceices*

1. INTRODUCTION

Somatic cell nuclear transfer (SCNT) is a crucial technique biomedical research and applications, including animal production, biotechnology, human xenotransplantation, and animal disease models (Yang, et al. 2007). However, the efficiency of mammalian SCNT requires improvement because the success rate is low (Whitworth and Prather 2017). One of the fundamental causes of these challenges is the quality of embryos produced *in vitro*. Therefore, to potentially obtain embryos with high developmental potential, it is essential to establish a stable *in vitro* maturation (IVM) system that produces high-quality oocytes. During IVM, oxidative stress is elevated in oocytes due to various environmental factors, including temperature variations (Nabenishi, et al. 2012), the gas atmosphere (Pinyopummintr and Bavister 1995), and alterations in the composition of the culture medium (Wang, et al. 1997). Oxidative stress primarily occurs when there is an imbalance between the production and neutralization of reactive oxygen species (ROS). Consequently, it impairs subsequent embryo development, ultimately diminishing reproductive success. Although antioxidant enzymes, such as catalase (*CAT*) and glutathione (GSH), are abundant in the female reproductive system, oocytes maturing *in vitro* lack protective antioxidant mechanisms (Agarwal, et al. 2006; Combelles, et al. 2009). Therefore, antioxidant treatment during IVM is an effective strategy to enhance the developmental capacity of oocytes by maintaining physiological ROS levels.

Ellagic acid (EA) is a natural polyphenol found in raspberries, strawberries, grapes, and nuts. Polyphenolic compounds are plant metabolites that exhibit potent antioxidant activity (Olszowy 2019; Pandey and Rizvi 2009). EA contains four hydroxyl groups and two lactone groups, which are responsible for its excellent antioxidant properties. EA has various biological functions such as antioxidant (Kilic, et al. 2014), anti-inflammatory (Wang, et al. 2022), anti-cancer (Mishra and Vinayak 2014) properties, as demonstrated in both *in vivo* and *in vitro* models. In particular, EA enhances the nuclear factor erythroid 2-related factor 2 (*Nrf2*) pathway, which can protect various cell types against inflammation or oxidative stress (Wang, et al. 2022). Furthermore, EA inhibits proliferation of cancer cells by suppressing activation of protein kinase C, which play critical roles in cell proliferation and tumor growth (Mishra and Vinayak 2014). Based on its biological activity, there is increasing interest in whether EA treatment can protect germ cells. Treatment of benzene-exposed human sperm with EA protects DNA integrity and enhances sperm vitality and motility by alleviating generation of intracellular ROS (Iovine, et al. 2021). EA prevents a decrease in sperm count, maintains sperm motility and viability, and decreases the incidence of sperm malformations in diabetic rats (ALTamimi, et al. 2021). Furthermore, Mottola *et al.* reported that EA enhances zebrafish embryo development and improves morphological characteristics by scavenging hydrogen peroxide (Mottola, et al. 2020). Although the bioprotective properties of EA are well-documented, its potential beneficial effects on porcine oocytes remain unclear.

In this study, we hypothesized that EA treatment during IVM may protect porcine oocytes by reducing the ROS levels and apoptosis. To confirm the optimal concentration of EA, we initially examined the developmental potential of porcine embryos upon parthenogenesis following treatment of oocytes with various concentrations of EA during IVM. The effects of EA on spindle morphology, mitogen-activated protein kinase (MAPK) activity, and expression of key genes related to embryo quality were further investigated. To elucidate the mechanisms by which EA protects oocytes, we assessed the levels of ROS and GSH and expression of antioxidant genes. Finally, we assessed the development and quality of embryos derived from these oocytes following SCNT. Our findings suggest EA protects oocytes and thus is a candidate to improve the efficiencies of ARTs.

2. Materials & Methods

2.1. Chemicals and reagents

In this study, all chemicals and reagents were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise.

2.2. Collection of porcine cumulus-oocyte complexes (COCs) collection and IVM

Ovaries obtained from locally slaughtered pigs were transported to the laboratory within 2 h at 30 - 33°C in a thermos in the presence of saline solution supplemented with 75 μg/L penicillin G and 50 μg/L streptomycin sulfate. Blood was removed from ovaries with saline solution and COCs were extracted from follicles with a diameter of 1 - 8 mm using an 18-gauge needle and a 10 mL syringe. COCs were washed three times each in tissue culture medium (TCM)-199-HEPES containing 0.1% (w/v) bovine serum albumin (BSA) and TCM-199 (M-199; Gibco, Grand Island, NY, USA) containing Earle's salts, 0.57 mM cysteine, 10 ng/mL epidermal growth (E-9644), 0.5 μg/mL follicle-stimulating hormone (F-2293), 0.5 μg/mL luteinizing hormone (L-5269), 10% (v/v) porcine follicular fluid, and different concentrations of EA (0, 0.1, 1, 10, and 100 μM). Oocytes surrounded by three layers of cumulus cells are collected. Based on this, selected COCs were matured in 500 μL of TCM-199 under mineral oil for 38 or 44 h at 38.8°C in a humidified atmosphere of 5% CO_2 and 95% air.

2.3. PA and *in vitro* culture

After IVM for 44 h, cumulus cells were removed from COCs by pipetting 40 - 50 times in 1 mg/mL hyaluronidase and oocytes were washed three times in TCM-199-HEPES. PA was performed with 5 μM Ca^{2+} ionomycin (Sigma) for 5 min. After culture for 3 - 4 h in porcine zygote medium-5 (PZM-5) containing 7.5 μg/mL cytochalasin B (CB, Sigma), activated oocytes were washed three times in PZM-5 containing 0.4% (w/v) BSA and cultured in the same medium for 7 days at 38.8°C in a humidified atmosphere of 5% CO_2 and 95% air. Activated oocytes were washed in Dulbecco's phosphate-buffered saline (DPBS) and either fixed in 4.0% (w/v) paraformaldehyde for 20 min and stored at 4°C, or snap-frozen in liquid nitrogen at -196°C and stored at −80°C.

2.4. SCNT and *in vitro* culture

After IVM for 38 h with or without 10 μM EA, cumulus cells were removed from COCs by pipetting 40 - 50 times in the presence of 1 mg/mL hyaluronidase and oocytes were washed three times in TCM-199-HEPES. The first polar body and nucleosome were removed using a 20 - 25 μm glass pipette while holding the oocyte with a 100 - 120 μm glass pipette in TCM-199-HEPES supplemented with 0.4% (w/v) BSA and 7.5 μg/mL CB with the Oosight imaging system. Nuclear donor fibroblasts for SCNT were derived from Jeju Black cattle. These cells were cultured in Dulbecco's modified Eagle's medium (11995-065, Gibco) containing 10% fetal bovine serum (SH30084.03; HyClone, Logan, UT), 0.1 mM β-mercaptoethanol (21985-023, Gibco), and 1% penicillin/streptomycin (15140-22, Gibco). Passage 4 cells were cultured for 2 - 3 days until they reached confluency and then expanded by passage. The donor cell was injected into the enucleated

perivitelline space adjacent to the cytoplasm.

Karyoplast-cytoplast complexes were fused in fusion medium containing 0.3 M D-mannitol, 0.5 mM HEPES, 0.05% (w/v) BSA, 0.05 mM $CaCl_2$, and 0.1 mM $MgSO_4$. Injected donor cells were aligned to the northern wire in a fusion chamber (Lf201; Nepagene, Chiba, Japan) with a direct current impulse of 105 V/cm for 60 μsec to induce fusion and electrical activation. Afterward, fused embryos were activated in 7.5 μg/mL CB for 4 h. SCNT embryos were transferred to PZM-5 containing 0.4% (w/v) BSA and cultured in the same medium for 7 days at 38.8°C in a humidified atmosphere of 5% CO_2 and 95% air.

2.5. Immunofluorescence staining

After IVM for 44 h with or without 10 μM EA, cumulus cells were removed from porcine COCs, and oocytes were fixed overnight at 4°C. Fixed oocytes were incubated for 30 min at 38.8°C with 0.5% (v/v) Triton X-100. After blocking for 1 h with 1% BSA (w/v) prepared in PBS (blocking solution I), oocytes were incubated overnight at 4°C with an Alexa Fluor 488-conjugated anti-α-tubulin antibody (Sigma, 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 onto glass slides, and examined under an inverted Olympus IX-71 microscope. Grayscale images were acquired on a microscope equipped with a digital camera. Mean grayscale values were determined using ImageJ software (NIH). The experiment was independently repeated four times with 15 - 20 oocytes per experiment.

2.6. Determination of intracellular ROS and GSH levels

The intracellular levels of ROS and GSH were measured using dichlorohydrofluorescein diacetate (DCFHDA) and CellTracker™ Blue 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CMF_2HC), respectively, as previously described (Yang, et al. 1998; You, et al. 2010) with slight modifications. In brief, denuded oocytes were incubated in DPBS containing 50 μM DCFHDA or 100 μM CellTracker™ Blue CMF_2HC in the dark for 20 min at 38.8°C. Thereafter, oocytes were washed more than five times in DPBS containing 0.1% (w/v) BSA to completely remove excess dye and promptly examined by epifluorescence microscopy (Olympus, Japan). The ROS level was determined using excitation and emission wavelengths of 450 - 490 nm and 515 - 565 nm, respectively. The excitation and emission wavelengths of GSH staining were 371 and 464 nm, respectively. Grayscale images were acquired with a digital camera (Nikon) attached to the microscope. Mean grayscale values were calculated using ImageJ software. Background fluorescence values were subtracted from the final values before statistical analysis and normalized to those of control oocytes. The experiment was independently repeated four times with ten oocytes per experiment.

2.7. TUNEL assay and Hoechst staining

On day 7 after PA or SCNT, BLs were fixed, 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. Fixed embryos were incubated with fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase (In Situ Cell Death Detection Kit; Roche, Manheim, Germany) in the dark for 1 h at 38.8°C. Nuclei were stained with Hoechst 33342 (1 μg/mL) for 30 min, and stained BLs were washed with PBS containing 0.1% BSA. Stained BLs were

mounted onto glass slides and examined under an inverted Olympus IX-71 fluorescence microscope. Mitotic and apoptotic cells were scored.

2.8. mRNA extraction and complementary DNA synthesis

mRNA was extracted from 20 - 30 oocytes or 10 - 15 BLs per replicate using a Dynabeads mRNA Direct Kit (Invitrogen, USA). Eluted mRNA was collected in 10 μL of elution buffer provided with the kit and then reverse-transcribed into complementary DNA using an oligo (dT) 20 primer and SuperScript II reverse transcriptase (Invitrogen) in accordance with the manufacturer's instructions.

2.9. Real-time quantitative polymerase chain reaction

Real-time RT-PCR was performed as previously described (Lee, et al. 2012) using the primer sets listed in Table 1 and a StepOnePlus Real-time PCR System (Applied Biosystems, USA) with a final reaction volume of 20 μL containing SYBR Green PCR Master Mix (Applied Biosystems). The conditions were as follows: 10 min at 95°C, followed by 39 cycles of 15 s at 95°C, 60 s at 54°C, 15 s at 95°C, and 60 s at 60°C. Samples were then cooled to 12°C for 5 min. Relative gene expression levels were analyzed by the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen 2001) after normalization against the expression level of a housekeeping gene (*ACTB*). The experiment was independently repeated three times.

Table 1. Primers used for real-time PCR

Gene	GenBank accession no.	Primer sequence*	Annealing temperature (°C)	Product size (bp)
ACTB	AF017079	F: GGGCATGAACCATGAGAAGT R: AAGCAGGGATGATGTTCTGG	60	230
Nrf2	XM_005671981.2	F: ACAACTCAGCACCTTGTACC R: CCTTACTCTCCAAGTGAGTACTC	54	81
HO-1	NM001004027.1	F: ACCCAGGACACTAAGGACCA R: CGGTTGCATTCACAGGGTTG	54	227
CAT	NM_214301	F: ACGTTGGAAAGAGGACACCC R: TCCAACGAGATCCCAATTACCA	54	137
SOD1	GU9444822.1	F: GCCACTGTGTACATCGAAGAT R: GTGATCCCAATTACACCACAG	54	173
SOD2	NM_214127.2	F: AGACCTGATTACCTGAAAGC R: CTTGATGTACTCGGTGTGAG	54	110
CDX2	AM778830	F: AGCCAAGTGAAAACCAGGAC R: TGCGGTTCTGAAACCAGATT	60	178
SOX2	EU503117	F: GCCCTGCAGTACAACTCCAT R: GCTGATCATGTCCCGTAGGT	60	216
POU5F1	NM_001113060	F: AGTGAGAGGCAACCTGGAGA R: TCGTTGCGAATAGTCACTGC	60	166
BCL2L1	NM_214285.1	F: GGTTGACTTTCTCTCCTACA R: CTCAGTTCTGTTCTTCCA	54	118
BAK	XM_001928147	F: GTACGCAGATTCTTCAGGTC R: AAAGTCCATAAAGGGGTCTC	60	70
FAS	AJ001202.1	F: GAGAGACAGAGGAAGACGAG R: CTGTTCAGCTGTATCTTTGG	54	194
CASP3	NM_214131	F: GAGGCAGACTTCTTGTATGC R: CATGGACACAATACATGGAA	55	236

*F, forward; R, reverse.

2.10. Western blot analysis

The protocol was basically the same as that described previously (Lee, et al. 2012). Briefly, oocytes (25 - 30 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 placed in a heating block for 5 min at 95°C. Proteins were resolved on 5 - 12% Tris SDS-polyacrylamide electrophoresis gels for 1.5 h at 80 - 120 V. Samples were then transferred to HybondECL nitrocellulose membranes at 400 mA for 1 h in transfer buffer (25 mM Tris, pH 8.5, containing 200 mM glycine and 20% [v/v] methanol). After blocking with 5% (w/v) skim milk prepared in PBS for 1 h, the membranes were incubated for at least 2 h with an anti-p44/42 MAPK or anti-phospho-p44/42 MAPK antibody diluted 1:300 in blocking solution (1× Tris-buffered saline, pH 7.5, containing 0.1% [v/v] Tween-20, and 5% [w/v] skim 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 h with anti-rabbit IgG-horseradish peroxidase diluted 1:2000 in blocking solution. After washing with TBST, immunoblots were visualized with a chemiluminescent reagent (Invitrogen). The experiment was independently repeated four times.

2.11. Statistical analysis

Data were evaluated using the general linear model procedure within the Statistical Analysis System (SAS User's Guide, 1985, Statistical Analysis System Inc.). Tukey's multiple range test was used to determine significant differences. Data are expressed as mean ± SEM. $p < 0.05$ was considered significant.

3. RESULTS

3.1. EA treatment during IVM of porcine oocytes improves subsequent embryo development following parthengenesis

To determine the optimal concentration of EA, oocytes were matured for 44 h in IVM supplemented with 0, 0.1, 1, 10, and 100 μM EA (control, 0.1 EA, 1 EA, 10 EA, and 100 EA groups, respectively). The percentage of oocytes that exhibited polar body emission was similar in each group (control, 80.5% ± 4.5%; 0.1 EA, 83.1% ± 3.4%; 1 EA, 87.3% ± 1.6%; 10 EA, 84.4% ± 3.0%; and 100 EA, 79.9% ± 2.2%; Figure 1A). Following parthenogenic activation (PA), the percentage of cleaved embryos on day 2 did not significantly differ among the control, 0.1 EA, 1 EA, and 100 EA groups, but was significantly higher ($p < 0.05$) in the 10 EA group than in the control group (control, 60.2% ± 2.5%; 0.1 EA, 61.0% ± 2.7%; 1 EA, 64.0% ± 2.7%; 10 EA, 69.8% ± 2.9%; and 100 EA, 60.0% ± 2.9%; Figure 1A). The percentage of cleaved embryos that reached the blastocyst (BL) stage on day 7 was significantly higher ($p < 0.01$) in the 10 EA group than in the control and 0.1 EA groups, but did not significantly differ between the control, 1 EA, and 100 EA groups (control, 22.2% ± 2.9%; 0.1 EA, 22.3% ± 2.1%; 1 EA, 28.3% ± 5.1%; 10 EA, 35.0% ± 3.7%; and 100 EA, 22.8% ± 4.4%; Figure 1A).

We evaluated BL quality by determining the total number cell per BL and the percentage of apoptotic cell in BLs on day 7. The total cell number per BL was significantly higher ($p < 0.05$) in the 10 EA group than in the control group, but did not significantly differ among the control, 0.1 EA, 1 EA, and 100 EA groups (control, 80.0 ± 4.5; 0.1 EA, 86.5 ± 8.0; 1 EA, 89.2 ± 6.7; 10 EA, 89.8 ± 2.9; and 100 EA, 80.3 ± 6.1; Figure 1B). Genomic DNA fragmentation was assessed by terminal deoxynucleotidyl transferase dUTP

nick end labeling (TUNEL) to detect apoptotic cells. The percentage of apoptotic cell in BLs was significantly lower ($p < 0.05$) in the 10 EA group than in the control group but did not significantly differ between the other groups (control, 2.6 ± 0.3 0.1 EA, 2.3 ± 0.5; 1 EA, 2.4 ± 0.6; 10 EA, 1.7 ± 0.3; and 100 EA, 2.7 ± 0.6; Figure 1C). Based on these results, the optimal concentration of EA was 10 μM. Subsequently, we conducted experiments using the control and 10 EA groups.

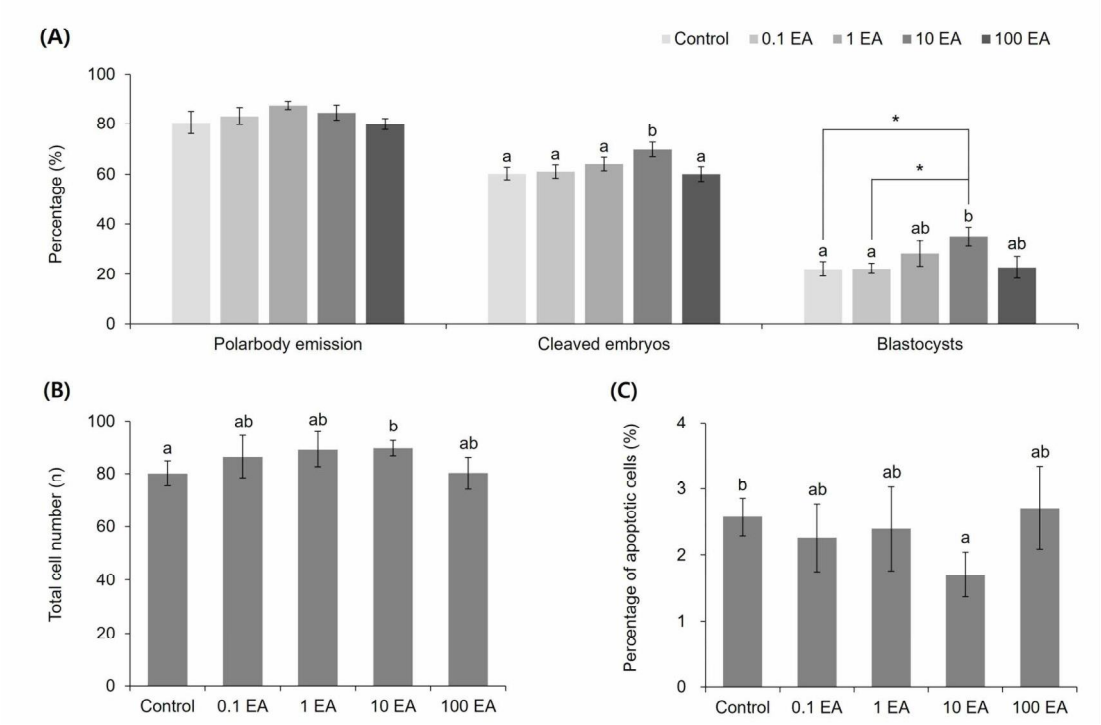

Figure 1. Effect of treatment with different concentrations of EA during IVM of porcine oocytes on embryos generated by PA. (A) Percentages of surviving oocytes, cleaved embryos on day 2, and blastocysts on day 7. (B) Total cell number per blastocyst on day 7. (C) Percentage of apoptotic cell in blastocysts on day 7. The experiment was independently repeated seven times. Values are presented as mean ± SEM of independent experiments ($^{a-b}p < 0.05$ and $^{*}p < 0.01$).

3.2. EA rescues abnormal spindle arrangement and chromosome alignment in porcine oocytes

To investigate the protective effect of EA during IVM of porcine oocytes, we examined spindle arrangement and chromosome alignment. We scored spindle and chromosome morphology as normal or abnormal as previously described (Lenie, et al. 2008) (Figure 2A). The percentage of oocytes with a normal meiotic spindle and normal chromosome alignment was significantly higher ($p < 0.01$) in the 10 EA group than in the control group (Figure 2B).

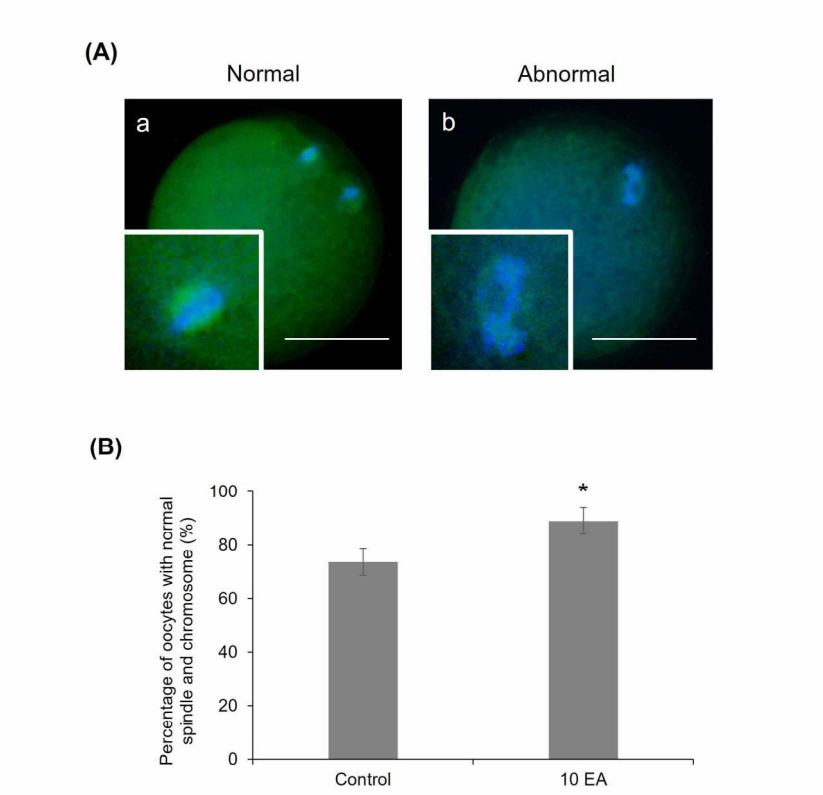

Figure 2. Effect of EA treatment during IVM on spindle arrangement and chromosome alignment in porcine oocytes. (A) Images of oocytes with normal and abnormal morphologies. Scale bar 100 μm. Magnification, 100×. (B) Percentage of oocytes with a normal spindle and chromosomes. The experiment was independently repeated seven times. Values are presented as mean ± SEM of independent experiments ($^{*}p < 0.01$).

3.3. EA increases the level of a cytoplasmic maturation factor in porcine oocytes

We investigated the effect of EA on cytoplasmic maturation. Active phospho-p44/42 MAPK migrated as a doublet in lysates of maturing porcine oocytes in western blot analysis (Figure 3A). The ratio of phospho-p44/42 MAPK to p44/42 MAPK was normalized against that in the control group. This ratio was significantly higher ($p < 0.001$) in the 10 EA group (1.48 ± 0.06) than in the control group (1.00 ± 0.0) (Figure 3B).

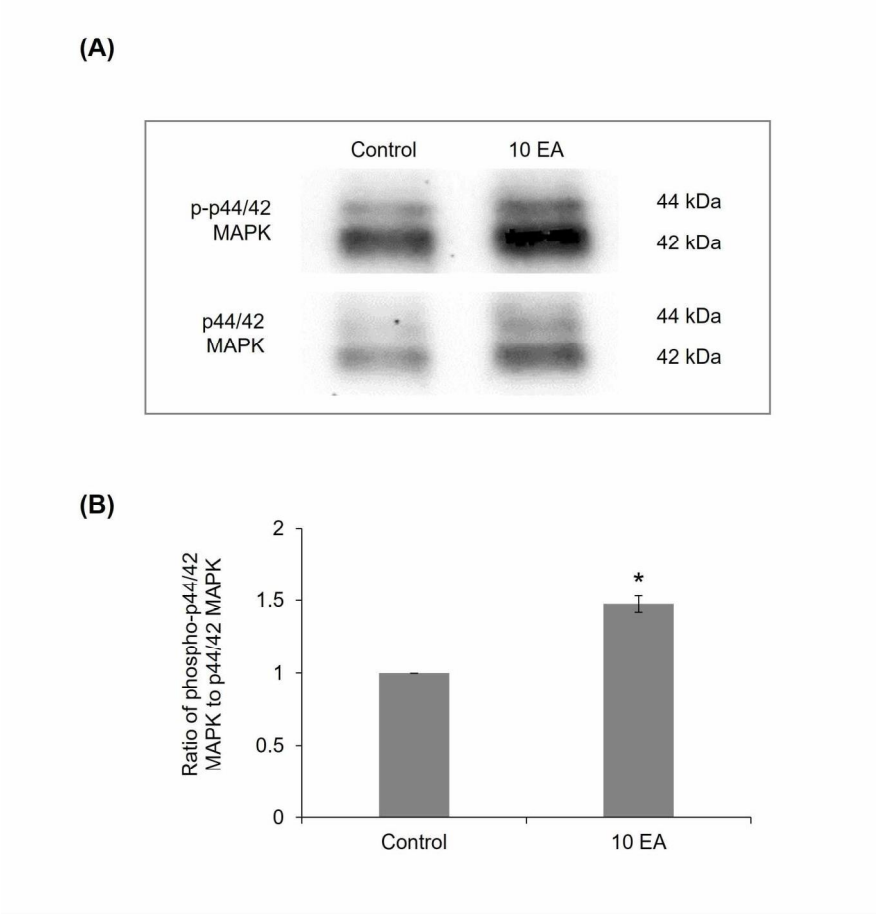

Figure 3. Effect of EA treatment during IVM on cytoplasmic maturation of porcine oocytes. (A) Western blot analysis of total and phosphorylated p44/42 MAPK. (B) Ratio of phospho-p44/42 MAPK to p44/42 MAPK. The experiment was independently repeated seven times. Values are presented as mean ± SEM of independent experiments ($^{*}p < 0.01$).

3.4. EA alleviates OS in porcine oocytes

To investigate the antioxidant effect of EA during IVM of porcine oocytes, we measured ROS and GSH levels and expression of antioxidant genes. The relative fluorescence intensity of ROS staining was significantly lower ($p < 0.01$) in the 10 EA group (0.93 ± 0.02) than in the control group (1.00 ± 0.0) (Figure 4A and B). The relative fluorescence intensity of GSH staining was significantly higher ($p < 0.001$) in the 10 EA group (1.05 ± 0.00) than in the control group (1.00 ± 0.0) (Figure 4A and 4C).

Expression of antioxidant genes (*Nrf2*, *CAT*, heme oxygenase-1 [*HO-1*], superoxide dismutase 1 [*SOD1*], and superoxide dismutase 2 [*SOD2*]) at the MII stage was analyzed by real-time RT-PCR (Figure 4D). Expression of *Nrf2* and *HO-1* was significantly higher ($p < 0.05$) in the 10 EA group than in the control group. Expression of *CAT* was significantly higher ($p < 0.001$) in the 10 EA group than in the control group. Expression of *SOD1* was significantly higher ($p < 0.01$) in the 10 EA group than in the control group. However, expression of *SOD2* was only slightly upregulated in the 10 EA group and did not significantly differ between the 10 EA and control groups.

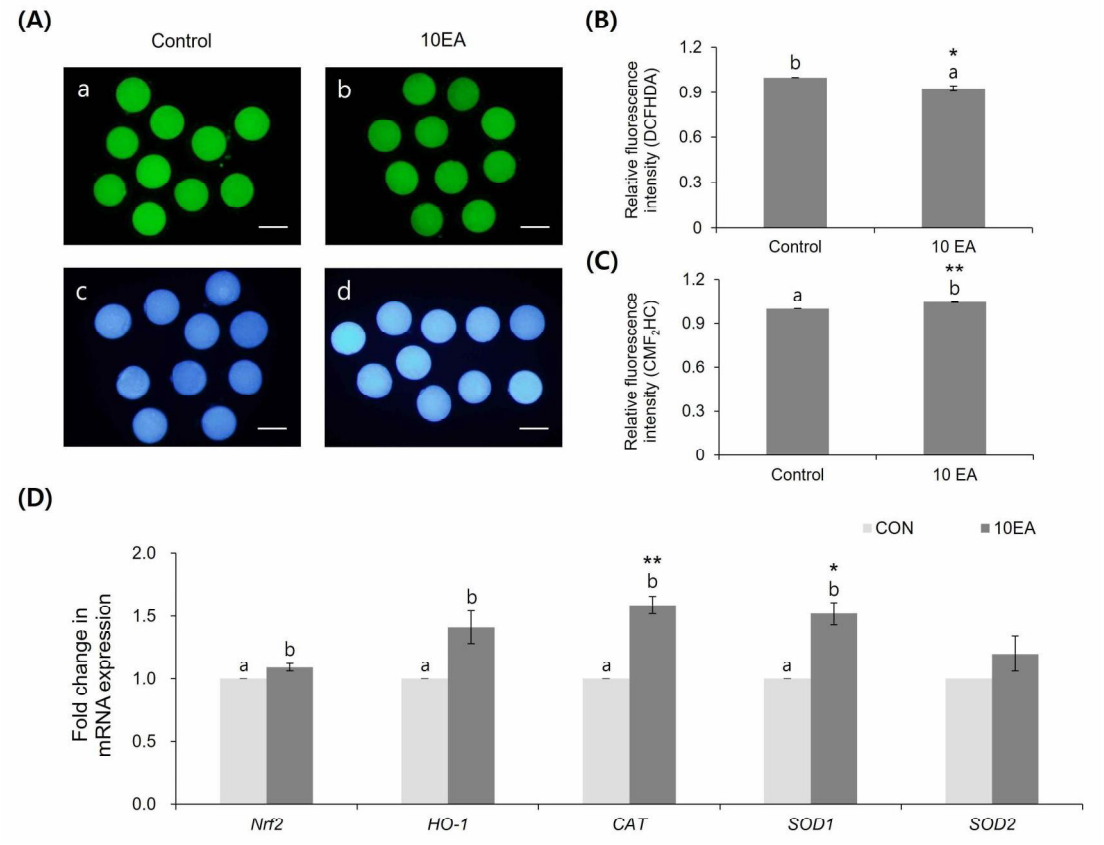

Figure 4. Antioxidant effect of EA treatment during IVM of porcine oocytes. (A) Images of oocytes stained with DCFHDA (green, a and b, ROS staining) and CMF_2HC (blue, c and d, GSH staining). Scale bar 100 μm. Magnification, 100×. (B) Quantification of the fluorescence intensity of DCFHDA. (C) Quantification of the fluorescence intensity of CMF_2HC. (D) Relative expression of the antioxidant genes *Nrf2*, *HO-1*, *CAT*, *SOD1*, and *SOD2*. ACTB was used as an internal standard. Data were normalized against the corresponding levels in the control group. The experiment was independently repeated seven times. Values are presented as mean ± SEM of independent experiments ($^{a-b}p < 0.05$, $^{*}p < 0.01$, and $^{**}p < 0.001$).

3.5. EA regulates expression of key genes related to embryo quality in porcine oocytes

The effect of EA treatment on expression of development-related (caudal type homeobox 2 [*CDX2*], POU domain, class 5, transcription factor 1 [*POU5F1*], and SRY-box transcription factor 2 [*SOX2*]) and apoptosis-related (BCL2-like 1 [*BCL2L1*], BCL2 antagonist/killer [*BAK*], fas cell surface death receptor [*FAS*], and cysteine-aspartic acid protease 3 [*CASP3*]) genes in parthenogenetic BLs at day 7 was investigated by qRT-PCR (Figure 5). Expression of the development-related genes *CDX2, POU5F1*, and *SOX2* was significantly higher ($p < 0.05$ or $p < 0.01$) in the 10 EA group than in the control group. Expression of the anti-apoptotic gene *BCL2L1* was significantly higher ($p < 0.05$) in the 10 EA group than in the control group. Expression of the pro-apoptotic genes *BAK, FAS*, and *CASP3* was significantly lower ($p < 0.001$ or $p < 0.01$) in the 10 EA group than in the control group.

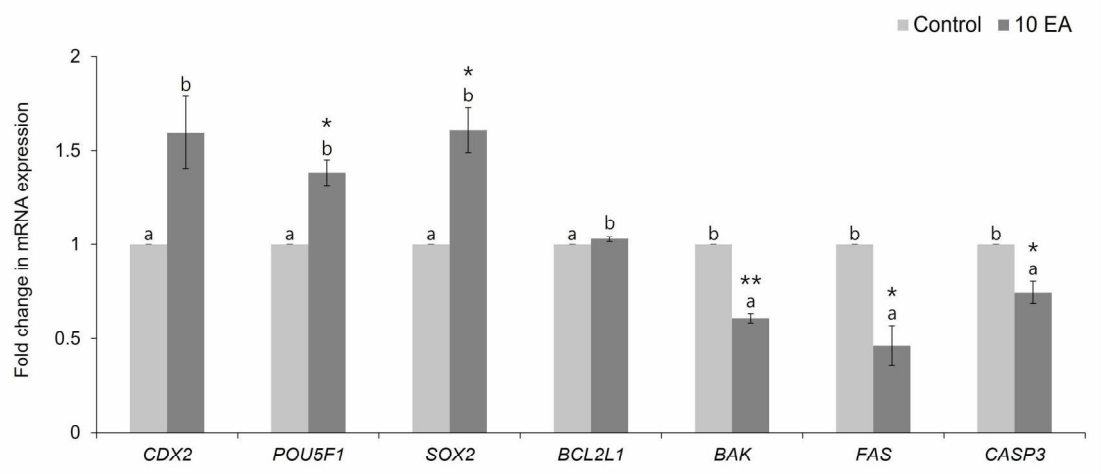

Figure 5. Effect of EA treatment during IVM of porcine oocytes on expression of key embryo quality-related genes. Relative mRNA expression of development related (*CDX2*, *POU5F1*, and *SOX2*), anti-apoptotic (*BCL2L1*), and pro-apoptotic (*BAK*, *FAS*, and *CASP3*) genes at the blastocyst stage. The experiment was independently repeated seven times. Values are presented as mean ± SEM of independent experiments ($^{a-b}p < 0.05$, $^{*}p < 0.01$, and $^{**}p < 0.001$).

3.6. EA treatment of porcine oocytes enhances the development and quality of embryos generated by SCNT

We determined the embryo development rates following SCNT to examine the effects of EA treatment on embryo development. The percentage of fused oocytes and cleaved embryos tended to be higher in the 10 EA group than in the control group, but there was no significant difference (control fused oocytes, 64.8% ± 1.0%; 10 EA fused oocytes, 65.9% ± 2.1%; control cleaved embryos, 62.6% ± 0.6%; and 10 EA cleaved embryos, 63.4% ± 5.1; Figure 6A). The percentage of BLs was significantly higher ($p < 0.05$) in the 10 EA group (23.5% ± 1.2%) than in the control group (20.5% ± 0.3%) (Figure 6A). Additionally, we assessed the quality of BL following SCNT by determining the total number of cells and the percentage of apoptotic cell in BLs on day 7. The total cell number per BL was higher in the 10 EA group (88.2 ± 1.0) than in the control group (83.5 ± 2.8), but there was no significant difference (Figure 6B). The percentage of apoptotic cell in BLs was significantly lower ($p < 0.05$) in the 10 EA group (1.8% ± 0.1%) than in the control group (2.8% ± 0.4%) (Figure 6C).

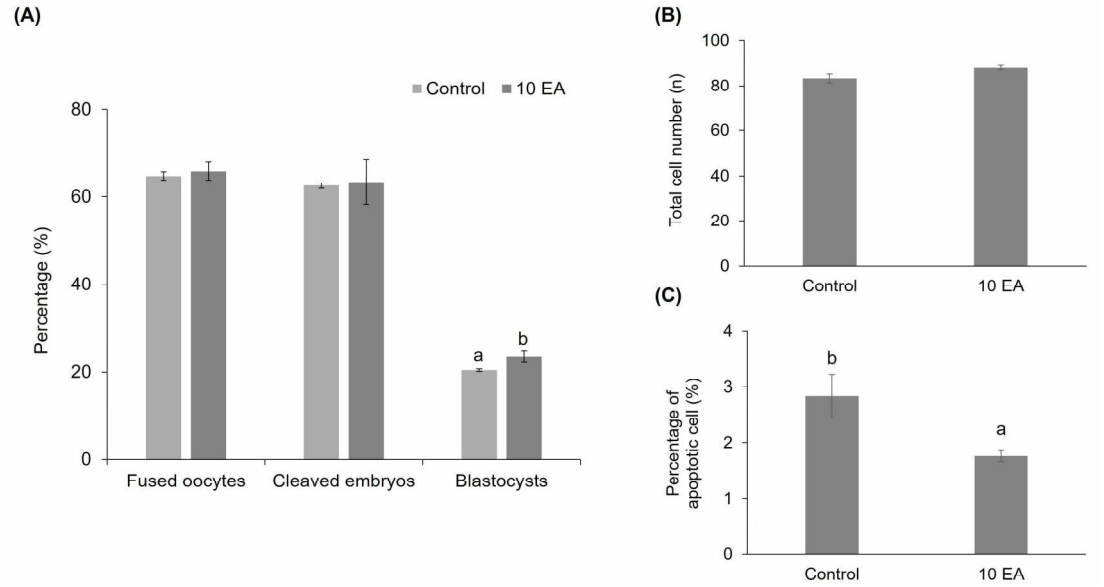

Figure 6. Effect of EA treatment on the development capacity of porcine oocytes after SCNT. (A) Percentages of fused oocytes, cleaved embryos on day 2, and blastocysts on day 7. (B) Total cell number per blastocyst on day 7. (C) Percentage of apoptotic cell in blastocysts on day 7. The experiment was independently repeated six times. Values are presented as mean ± SEM of independent experiments ($^{a-b}p < 0.05$, and $^{*}p < 0.01$).

4. DISCUSSION

Porcine IVM systems have significantly developed in recent years, leading to noticeable improvements in ARTs. These advances have enabled the successful birth of living piglets through techniques such as *in vitro* fertilization and SCNT using *in vitro* matured oocytes that have undergone IVM (Suzuki, et al. 2012). However, despite these achievements, IVM can directly influence various molecules and functions, reducing quality and developmental potential compared with *in vivo* maturation. Oxidative stress arises during IVM due to an imbalance between ROS and antioxidants, which impairs oocyte development (Agarwal, et al. 2006; Combelles, et al. 2009). In recent years, studies have reported that polyphenol-based antioxidants can reduce oxidative stress in oocytes in vitro. Resveratrol improves monospermic fertilization, blastocyst formation and blastomere viability of PA and IVF-derived embryos and protects porcine oocytes by reducing intracellular ROS (Kwak, et al. 2012). Additionally, supplementation of quercetin in Medium 199 medium alleviated autophagy and apoptosis in human and aged rat oocytes and reduced *SIRT3*, resulting in cellular antioxidant activity (Kang, et al. 2013). Hence, this study aimed to investigate the protective effect of EA during IVM of porcine oocytes.

Optimization of IVM systems improves the efficiency with which embryos are produced (Lonergan, et al. 2001). We investigated the effects of supplementation of IVM with EA, which has antioxidant

and anti-apoptotic activities. The cleavage and blastocyst formation rates were highest in the 10 EA group. In addition, the total cell number per blastocyst was highest and the percentage of apoptotic cell in blastocysts was lowest in this group. However, these beneficial effects were not observed in the 100 EA group. These findings were consistent with previous reports that higher concentrations of antioxidants can negatively affect oocyte maturation (Pyeon, et al. 2021; Wang, et al. 2014). While antioxidants play an important role in safeguarding oocytes during IVM, treatment with high doses of antioxidants can disrupt ROS-mediated signaling in oocytes, potentially impairing embryo development and quality. Our study demonstrates that treatment of porcine oocytes with 10 μM EA improves subsequent embryo development following parthenogenesis.

To assess whether EA improved subsequent embryo development by influencing meiotic maturation, we evaluated both nuclear and cytoplasmic maturation of oocytes. Cytoskeletal dynamics regulate nuclear maturation in oocytes (Wu, et al. 2022). Antioxidant defense can ensure normal chromosome segregation, which is essential for mammalian embryo development (Peng, et al. 2023). Unlike somatic cells, mammalian oocytes lack a centrosome during meiosis and therefore chromosomes are segregated by the spindle. Abnormal spindle formation can lead to non-segregation of chromosomes, resulting in production of abnormal oocytes including aneuploid oocytes (Namgoong and Kim 2018). The percentage of oocytes with normal spindles and chromosome alignment was significantly higher in the 10 EA group than in the control group. These results suggest that EA treatment

during IVM enhances nuclear maturation by protecting the spindle and ensuring normal chromosome alignment in MII oocytes.

Our results concerning MAPK activity are consistent with those concerning meiotic spindle formation. The MAPK pathway plays an important role in cytoplasmic maturation of vertebrate oocytes (Nebreda and Ferby 2000). ERK1/2 are components of the MAPK signaling pathway and participate in cytoplasmic maturation of oocytes (Kalous, et al. 2018). Inhibition of ERK1/2 activity reduces the embryo cleavage and blastocyst development rates (Ni, et al. 2015). The ratio of phospho-p44/42 MAPK to p44/42 MAPK was significantly increased in the 10 EA group. This suggests that EA treatment during IVM facilitates cytoplasmic maturation of porcine oocytes. Taken together, these results suggest that EA improves oocyte maturation and enhances subsequent embryo development.

While antioxidant enzymes, such as *CAT* and GSH, are abundant in the female reproductive system, oocytes maturing *in vitro* lack protective antioxidant mechanisms (Agarwal, et al. 2006; Combelles, et al. 2009). Therefore, addition of antioxidants to IVM medium enhances oocyte maturation and embryo development by regulating the redox balance (Pyeon, et al. 2021). We hypothesized that the effects of EA on oocyte maturation are associated with its antioxidant effects. To investigate this, we assessed the levels of ROS and GSH and expression of antioxidant-related genes. Porcine oocytes are particularly sensitive to ROS due to their abundant lipid contents in the cytoplasm (Dunning, et al. 2014). Intracellular GSH is the major

non-protein sulfhydryl compound in mammalian cells and protects oocytes from oxidative stress by neutralizing ROS (Zhou, et al. 2019). GSH is potentially an indicator of the effectiveness of EA as a free radical scavenger. EA scavenges radical species including hydroxyl, peroxyl, and nitrogen dioxide radicals (Priyadarsini, et al. 2002). The ROS and GSH levels were decreased and increased in the 10 EA group, respectively. These results are support by the previous findings that EA reduces ROS levels and prevents a decrease of GSH levels (Hwang, et al. 2010; Kim, et al. 2013). EA protects zebrafish embryos against hydrogen peroxide by reducing intracellular ROS levels and preventing DNA damage (Mottola, et al. 2020). In diabetic rats, supplementation of EA reduces ROS levels and increases GSH levels, thereby decreasing testicular damage and sperm abnormalities (ALTamimi, et al. 2021).

Expression of antioxidant genes (*Nrf2*, *HO-1*, *CAT*, *SOD1*, and *SOD2*) was upregulated in the 10 EA group than in the control group. Among these genes, *Nrf2* encodes a key regulator of endogenous antioxidant responses that alleviates oxidative damage and enhances cell viability (Ma, et al. 2018). It also plays a crucial role in transcriptional activation of genes encoding various antioxidants such as *HO-1, CAT,* and *SOD* (Dreger, et al. 2009). *HO-1* is upregulated by various stress conditions such as oxidative stress, inflammation, and ultraviolet radiation, and protects cells by catalyzing heme degradation (Consoli, et al. 2021). *CAT* catalyzes conversion of hydrogen peroxide into water and molecular oxygen (Liu and Kokare 2023), while *SOD1* converts superoxide radicals into hydrogen peroxide and molecular

oxygen (Allen and Tresini 2000). Additionally, gene expression of *SOD2*, which targets mitochondria and neutralizes superoxide radicals during cellular respiration (He, et al. 2019; Karnati, et al. 2013), did not significantly differ between the groups. EA upregulated the *Nrf2* pathway, which protects various cell types against inflammation, photoaging, and oxidative stress (ALTamimi, et al. 2021; Wang, et al. 2022). Additionally, EA upregulated antioxidant enzymes (*CAT*, *SOD*, *GSH*, and *GSH* peroxidase), which are downregulated by acetate in rat testis (Bidanchi, et al. 2022). Consistently, our results suggest that EA treatment during IVM improves oocyte maturation and embryo development by reducing the intracellular ROS level and activating the *Nrf2* pathway. Taken together, these findings demonstrate that EA effectively protects porcine oocytes through an antioxidant defense mechanism.

To investigate the effects of EA on blastocysts, we assessed expression of genes related to development and apoptosis. Development-related genes including *CDX2*, *POU5F1*, and *SOX2* were upregulated in the 10 EA group. Among these genes, *CDX2* is a specific indicator of trophectoderm (TE) (Strumpf, et al. 2005). TE forms the placenta, the outer tissue of the embryo, while the inner cell mass (ICM) influences formation of the hypoblast and epiblast. Separation of TE from the ICM in the embryo is pivotal during blastocyst formation. While *CDX2* is involved in separation of the ICM and TE, *POU5F1* and *SOX2* are important for maintaining cell fate in the ICM and serve as key regulators of pluripotency, which is essential for early embryo development (Cauffman, et al. 2004; Chambers, et al.

2003; Masui, et al. 2007). Given that downregulation of *POU5F1* during embryo development can disrupt ICM formation (Nichols, et al. 1998), its upregulation may improve early embryo development. Taken together, these data show that a correct gene expression is essential for high-quality embryo production.

Apoptosis, also referred to as programmed cell death, is affected by oxidative stress and plays an important role in early mammalian embryo development. Accordingly, it is essential to regulate the balance between anti- and pro-apoptotic genes with antioxidant treatment. EA elicits anti-apoptotic effects in the liver, kidneys, and testes by inhibiting activation of Bcl2-associated X protein [*BAX*] and *CASP3* and upregulating *BCL-2*, a key factor for cell survival (Bidanchi, et al. 2022). EA inhibits *CASP3* activation and subsequently reduces downstream *BAX* expression, and thereby elicits an anti-apoptotic effect on both liver and brain cells (Chen, et al. 2018). Anti-apoptotic (*BCL2L1*) and pro-apoptotic (*BAK, FAS,* and *CASP3*) genes were upregulated and downregulated in the 10 EA group, respectively. *BCL2L1* promotes cell survival by forming heterodimers with pro-apoptotic BCL-2 family proteins, preventing mitochondrial outer membrane disruption and cytochrome c release (White 1996). By contrast, when pro-apoptotic *BAK* is activated, it triggers mitochondrial outer membrane permeabilization, resulting in release of cytochrome c into the cytoplasm and ultimately activating the apoptotic pathway (Renault, et al. 2013; Yuan and Akey 2013). Meanwhile, the *FAS* pathway, which is initiated by binding to *FAS* ligand, culminates in *CASP3* activation through formation of the death-inducing signal

complex (Goillot, et al. 1997). When *CASP3* is activated, it degrades key matrix proteins and induces morphological and biochemical changes that are hallmarks of apoptosis, including DNA fragmentation and chromosome condensation (Enari, et al. 1998; Liu, et al. 1997). Our results suggest that EA treatment upregulates development-related genes and concurrently alters expression of apoptosis-related genes. Therefore, EA may improve embryo quality by regulating molecular mechanisms.

The effects of EA, which improved oocyte maturation and subsequent embryo development following PA, were also assessed in embryos generated by SCNT. SCNT involves removal of nuclei from oocytes and injection of somatic cell nuclei, which may disrupt the normal pathways responsible for embryo development (Srirattana, et al. 2022). Antioxidant treatment reportedly improves embryo development after SCNT (Jin, et al. 2016; Wang, et al. 2019). Therefore, we assessed the development and quality of embryos generated by SCNT to confirm the protective effect of EA. The blastocyst formation rate was increased and the percentage of apoptotic cell in blastocysts was decreased in the 10 EA group. These results suggest that the antioxidant EA elicits beneficial effects on porcine oocytes following PA and can improve the efficiency of SCNT. To optimize the SCNT protocol using EA, additional studies of nuclear reprogramming and transgenic animal production are needed.

In summary, our study demonstrated that EA treatment during IVM improves subsequent embryo development. Specifically, EA

improved both nuclear and cytoplasmic maturation through an antioxidant mechanism. Moreover, EA enhanced the quality of blastocysts by regulating genes related to development and apoptosis. Furthermore, EA improved the formation and quality of blastocysts generated following SCNT. These findings offer new insights into the protective role of EA against oxidative stress and are expected to be valuable for ARTs.

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엘라그산이 돼지 난모세포의 체외 성숙 동안 처녀생식과 체세포 핵 치환 이후 발달 능력에 미치는 영향

이한비

제주대학교 대학원 분자생명공학전공

엘라그산은 천연 폴리페놀이자 항산화 특성을 지닌 자유 라디칼 제거제이다. 본 연구는 돼지 난모세포의 체외 성숙 동안 EA의 보호 효과를 조사하였다. 최적의 농도를 결정하기 위해 IVM 배지에 다양한 농도의 EA가 보충되었다. 10 μM EA(10 EA)로 처리하면 분열률, 배반포 형성률, 배반포당 총 세포 수가 가장 높았고 세포사멸 비율이 가장 낮았다. 10 EA 그룹에서는 비정상적인 방추와 염색체 오정렬이 완화되었고 전체 p44/42에 대한 인산화된 p44/42의 비율이 증가하였다. 또한 10 EA 그룹에서 활성산소종과 글루타티온 수치가 각각 유의하게 감소 및 증가하였고 항산화 유전자(*Nrf2, HO-1, CAT* 및 *SOD1*)도 유의하게 상향 조절되었다. 발달 관련(*CDX2, POU5F1* 및 *SOX2*) 및 항세포사멸 (*BCL2L1*) 유전자의 mRNA 발현은 10 EA 그룹에서 유의하게 상향 조절된 반면, 프로세포사멸 유전자(*BAK, FAS* 및 *CASP3*)의 mRNA 발현은 유의하게 하향 조절되었다. 궁극적으로 체세포 핵 이식에서는 BL 형성 속도가 크게 증가되었으며 세포사멸 비율은 10 EA 그룹에서 크게 감소되었다. 결론적으로, IVM 배지에 EA를 첨가하면 항산화 메커니즘을 통해 난모세포 성숙과 후속 배아 발달 능력이 향상시킨다는 것을 입증하였다. 이러한 발견은 EA가 보조 생식 기술의 효율성을 향상시킬 수 있음을 시사한다.

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