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Thesis for the degree of Master of Science

Piperine Improves the Quality of Porcine Oocytes during *In Vitro* Maturation by Reducing Oxidative Stress

Eun-Seo Lim

Department of Biotechnology

The Graduate School

Jeju National University

February 2024

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Piperine Improves the Quality of Porcine Oocytes during *In Vitro* Maturation by Reducing Oxidative Stress

A Thesis submitted to the graduate school of
Jeju National University in partial fulfillment of
requirements for the degree of Master of Science
under the supervision of **Se-Pill Park**

The thesis for the degree of Master of Science
by **Eun-Seo Lim**
has been approved by the dissertation committee.

February 2024

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Member	Eun-Young Kim	Eun Young Kim
Member	Se-Pill Park	Se Pill Park

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Piperine Improves the Quality of Porcine Oocytes during *In Vitro* Maturation by Reducing Oxidative Stress

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Oxidative stress caused by light and high temperature arises during *in vitro* maturation (IVM), resulting in low-quality embryos compared with those obtained *in vivo*. To overcome this problem, we investigated the effects of treatment with piperine (PIP) during maturation of porcine oocytes on subsequent embryo development *in vitro*. Porcine oocytes were cultured in IVM medium supplemented with 0, 50, 100, 200, or 400 μM PIP. After parthenogenetic activation, the blastocyst formation rate was significantly higher and the percentage of apoptotic cells in blastocysts was significantly lower using 200 μM PIP-treated oocytes (200 PIP). In the 200 PIP group, the level of reactive oxygen species at the metaphase II stage was decreased, accompanied by an increased level of glutathione and increased expression of antioxidant genes (*Nrf2*, *CAT*, *HO-1*, *SOD1*, and *SOD2*). Consistently, chromosome misalignment and aberrant spindle organization were alleviated and phosphorylated p44/42 mitogen-activated protein kinase activity was increased in the 200 PIP group. Expression of development-related (*CDX2*, *NANOG*, *POU5F1*, and *SOX2*), anti-apoptotic (*BCL2L1* and *BIRC5*), and pro-apoptotic (*BAK*, *FAS*, and *CASP3*) genes was altered in the 200 PIP group. Embryo development following somatic cell

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nuclear transfer was improved in the 200 PIP group. These findings suggest that PIP improves the quality of porcine oocytes by reducing oxidative stress, which inevitably arises during IVM. In-depth mechanistic studies of porcine oocytes will improve the efficiencies of assisted reproductive technologies.

Key words: *Piperine*, *Antioxidant*, *IVM*. *SCNT*, *Oocytes*

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1. INTRODUCTION

In vitro maturation (IVM) techniques for porcine oocytes involving regulation of oxidative stress may facilitate human reproduction. Pigs have anatomical and physiological similarities with humans and are therefore used to study human diseases (Aigner, et al. 2010; Lunney 2007). Assisted reproductive technologies (ARTs), including *in vitro* production and somatic cell nuclear transfer (SCNT), are important to develop models of diseases (Dai, et al. 2002; Lai, et al. 2002). Among ARTs, IVM is essential because it involves maturation of oocytes from the first meiosis to the metaphase II (MII) stage. This is critical for subsequent development of embryos produced *in vitro* (Hatırnaz, et al. 2018). However, early porcine embryos are particularly sensitive to oxidative stress during *in vitro* production due to their unique structural characteristics (Kikuchi, et al. 2002; McEvoy, et al. 2000). Oxidative stress caused by reactive oxygen species (ROS) has detrimental effects on oocytes, such as induction of mitochondrial dysfunction, DNA fragmentation, apoptosis, and cytoplasmic abnormalities (Agarwal, Durairajanayagam and Du Plessis 2014; Agarwal, et al. 2006; Bansal and Bilaspuri 2011; Morado, et al. 2009). Accordingly, it has been reported that the capacity and quality of embryo development are lower *in vitro* than *in vivo* (Nakamura, Tajima and Kikuchi 2017; Zhao, et al. 2020). Recent studies showed that treatment of porcine oocytes with antioxidants enhances the developmental capacity and quality of blastocysts (BLs) by maintaining the intracellular redox equilibrium (Kim, et al. 2019; Pyeon, et al. 2021; Yoon, et al. 2021). Treatment with antioxidants may be an effective strategy to improve the quality of oocytes during IVM.

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Black pepper (*Piper nigrum* L.) is a spice used worldwide and a major medicinal plant. Pepper contains various compounds such as alkaloids, flavonoids, and phenols (Agbor, et al. 2006). The spicy taste and major physiological properties of black pepper are due to the alkaloid piperine (PIP) (Meghwal and Goswami 2013; Srinivasan 2007). PIP has diverse physiological effects, including antioxidant, anti-inflammatory, anti-cancer, and anti-obesity effects (Damanhouri and Ahmad 2014; Stojanović-Radić, et al. 2019). Although PIP can contribute to obesity regulation by improving insulin and leptin sensitivity (BrahmaNaidu, et al. 2014) and exhibits cytotoxic effects on lung cancer cells by arresting them in G2/M phase of the cell cycle (Lin, et al. 2014), its most noteworthy attribute is its antioxidant effects. The antioxidant effects of PIP can regulate lipid peroxidation (Selvendiran, et al. 2003) and contribute to anti-inflammatory and immunomodulatory activities via inhibition of NF-κB activity (Bang, et al. 2009). Additionally, PIP reportedly protects against oxidative damage by reducing ROS levels (Srinivasan 2007), but its effect on porcine oocytes has not been studied. We expected PIP to have an antioxidant capacity during IVM of porcine oocytes.

Given the necessity of antioxidants for IVM of porcine oocytes, this study investigated the influence of PIP treatment during IVM of porcine oocytes on subsequent embryo development *in vitro*. We specifically focused on antioxidant effects, which are closely associated with the nuclear and cytoplasmic maturation efficiency of porcine oocytes. Additionally, we evaluated the developmental competence and quality of BLs obtained following parthenogenesis and SCNT. The findings of this study suggest that PIP has the potential to enhance the quality of embryos by eliciting antioxidant effects *in vitro*.

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2. Materials & Methods

2.1. Chemicals and reagents

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

2.2. Oocyte collection and IVM

Prepubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory within 2 h. During transport, they were immersed in saline solution containing 75 μg/mL penicillin G and 50 μg/mL streptomycin sulfate and maintained at 30 - 33°C. Cumulus-oocyte complexes (COCs) were aspirated from follicles with a diameter of 2 - 8 mm. COCs were washed three times in tissue culture medium (TCM)-199 - HEPES containing 0.1% (w/v) bovine serum albumin (BSA). Thereafter, 50 COCs per group was treated with 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. Maturation was performed using mineral oil at 38.8°C for 44 h in an atmosphere of 5% CO_2 and 95% air. During IVM, the medium was supplemented with 0, 50, 100, 200, or 400 μM PIP. PIP was dissolved in 1% dimethyl sulfoxide (DMSO) as a 100 × stock for each group and frozen until use. The 0 PIP group was treated only with the solvent, DMSO.

2.3. PA and embryo culture

After 44 h of IVM, porcine oocytes were denuded of cumulus cells by

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pipetting 40 times in TCM-199-HEPES containing 0.1% hyaluronidase. Oocytes with the first polar body (PB) at MII stage were placed in PA solution (5 μM Ca^{2+} ionomycin [Sigma]) for 5 min and incubated in porcine zygote medium (PZM)-5 containing 7.5 μg/mL cytochalasin B (Sigma) for 3.5 h. Denuded oocytes were washed in PZM-5 containing 0.4% (w/v) BSA three times and cultured for 7 days at 38.8°C in a humidified atmosphere of 5% CO_2 and 95% air. The rate of BL formation was calculated as the ratio of BL formation to cleavage at day 2. The blastocyst formation was judged by the formation of the blastocoel, its size increasing, and the zona pellucida becoming thinner. Denuded oocytes and PA embryos were washed in washing solution (Dulbecco's phosphate-buffered saline [DPBS] containing 0.1% BSA), and, depending on the experiment, either fixed in fixing solution (4.0% [w/v] paraformaldehyde) and stored at 4°C or snap-frozen in liquid nitrogen and stored at −80°C.

2.4. TUNEL assay and Hoechst staining

Fixed BLs were washed more than four times in washing solution and incubated with 0.1% Triton X-100 at 38.8°C for 30 min. BLs 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 for 30 min. Stained BLs were washed in washing solution. Washed BLs were mounted onto glass slides and examined under an inverted Olympus IX-71 fluorescence microscope.

2.5. Measurement of intracellular ROS and GSH levels

Dichlorohydrofluorescein diacetate (DCFHDA) and CellTracker™ Blue

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4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CMF_2HC) were used to measure the intracellular levels of ROS and GSH, respectively, as previously described (Yang, et al. 1998; You, et al. 2010) with slight modifications. Briefly, 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 washing solution to completely remove excess dye and immediately analyzed by epifluorescence microscopy (Olympus, Japen). The ROS level was determined using excitation and emission wavelengths of 450 - 490 nm and 515 - 565 nm, respectively. The GSH level was determined using excitation and emission wavelengths of 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.

2.6. Immunofluorescence

Oocyte meiotic spindles and nuclei were visualized after maturation. Denuded oocytes were fixed overnight at 4°C in fixing solution. Fixed oocytes were incubated for 30 min at 38.8°C with 0.5% (v/v) Triton X-100. Permeabilized oocytes were blocked for 1 h with 1% BSA (w/v) prepared in phosphate-buffered saline (PBS, blocking solution I) and 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 for 30 min. Stained oocytes were washed three times in washing solution. Washed oocytes were mounted onto glass slides and examined under an inverted Olympus IX-71 microscope. Grayscale images were acquired using a microscope equipped with a digital camera. Mean grayscale values were

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calculated using ImageJ software (NIH). At least 15 oocytes were examined per group.

2.7. Extraction of messenger RNA (mRNA) and synthesis of complementary DNA

mRNA was isolated from three biological replicates using a Dynabeads mRNA Direct Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Isolated mRNA was collected in 10 μL of elution buffer provided with the kit. Subsequently, eluted RNA was reverse-transcribed into cDNA using an oligo (dT) 20 primer and SuperScript II reverse transcriptase (Invitrogen). At least 30 oocytes or 15 BLs were examined per group.

2.8. Real-time reverse transcription polymerase chain reaction

The protocol used was basically the same as that described previously (Lee, et al. 2012). Real-time RT-PCR was performed 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 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. Samples were then cooled to 12°C. Relative gene expression was analyzed using the $2-\Delta\Delta Ct$ method (Livak and Schmittgen 2001) after normalization against expression of a housekeeping gene (*ACTB*).

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Table 1. Primers used for qPCR

Gene	GenBank accession no.	Primer sequences	Annealing temperature (°C)	Product size (bp)
ACTB	AY550069.1	F: AGATCATGTTCGAGACCTTC R: GTCAGGATCTTCATGAGGTAGT	54	220
CAT	NM_214301.2	F: AGGGAGAGGCGGTTTATTGC R: GGACTCGTTGGTGAAGCTCA	59	117
HO-1	NM_214127.2	F: ACCCAGGACACTAAGGACCA R: CGGTTGCATTCACAGGGTTG	52	227
NFE2L2	XM_005671981.2	F: ACAACTCAGCACCTTGTACC R: CCTTACTCTCCAAGTGAGTACTC	54	81
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
NANOG	DQ447201	F: TTCCTTCCTCCATGGATCTG R: ATCTGCTGGAGGCTGAGGTA	60	214
POU5F1	NM_001113060	F: AGTGAGAGGCAACCTGGAGA R: TCGTTGCGAATAGTCACTGC	60	166
SOX2	EU503117	F: GCCCTGCAGTACAACTCCAT R: GCTGATCATGTCCCGTAGGT	60	216
BCL2L1	NM_214285.1	F: GGTTGACTTTCTCTCCTACA R: CTCAGTTCTGTTCTTCCA	54	118
BIRC5	NM_214141.1	F: CTTCTGCTTCAAAGAGCTG R: GGCTCTTTCTTTGTCCAGT	54	154
BAK	AJ001204	F: CTAGAACCTAGCAGCACCAT R: CGATCTTGGTGAAGTACTC	60	151
FAS	AJ001202.1	F: GAGAGACAGAGGAAGACGAG R: CTGTTCAGCTGTATCTTTGG	54	194
CASP3	NM_214131	F: GAGGCAGACTTCTTGTATGC R: CATGGACACAATACATGGAA	55	236

F, forward; R, reverse.

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2.9. Western blot analysis

The protocol was basically the same as that described previously (Lee, et al. 2012). Denuded oocytes 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 10% Tris SDS-polyacrylamide electrophoresis gels for 20 min at 80 V and 1 h at 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). The membranes were blocked with 5% (w/v) nonfat milk prepared in PBS for 2 h and incubated overnight at 4°C with an anti-p44/42 mitogen-activated protein kinase (MAPK) or anti-phospho-p44/42 MAPK antibody diluted 1:300 in blocking solution (PBS containing 5% [w/v] nonfat milk). Thereafter, the membranes were washed four times for 10 min in TBST (PBS containing 0.1% [v/v] Tween-20) and incubated for 1 h with anti-rabbit IgG-horseradish peroxidase diluted 1:2000 in blocking solution. After three washes with TBST, immunoreactive protein bands were visualized with a chemiluminescent reagent (Invitrogen).

2.10. SCNT and *in vitro* culture

After IVM for 36 - 38 h, porcine oocytes were denuded of cumulus cells by pipetting about 40 times in TCM-199-HEPES containing 0.1% hyaluronidase. The first polar body (PB) and nucleosome were removed from denuded oocytes using a 20 µm glass pipette under an Oosight Imaging System (Cambridge Research & Instrumentation, Inc., USA) in TCM-199-HEPES containing 0.4% (w/v) BSA and 7.5 µg/mL cytochalasin B.

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Donor fibroblasts for SCNT originated from Jeju Black cattle. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 0.1 mM β-mercaptoethanol, and 1% penicillin/streptomycin until they reached confluency and expanded by passage. The donor cell was injected into the perivitelline space surrounding the cytoplasm. Karyoplast-cytoplast complexes were fused in fusion medium (0.3 M D-mannitol, 0.5 mM HEPES, 0.05% [w/v] fatty acid-free BSA, 0.05 mM $CaCl_2$, and 0.1 mM $MgSO_4$) and exposed to a direct current of 101 V/cm for 60 μsec. Following fusion, activated embryos in the presence of 7.5 μg/mL cytochalasin B were transferred to PZM-5 supplemented with 0.4% (w/v) fatty acid-free BSA and incubated for 3.5h. These oocytes were washed in PZM-5 containing 0.4% (w/v) BSA three times and cultured for 7 days at 38.8°C in a humidified atmosphere of 5% CO_2 and 95% air. The rate of BL formation was calculated as the ratio of BL formation to cleavage at day 2. The blastocyst formation was judged by the formation of the blastocoel, its size increasing, and the zona pellucida becoming thinner. At least 70 oocytes were examined per group.

2.11. Statistical analysis

All experimental data were analyzed using the general linear model procedure provided by the Statistical Analysis System (SAS User's Guide, 1985, Statistical Analysis System Inc.). To assess significant differences, Tukey's multiple range test was employed, and significance was defined as a p-value < 0.05. Values are expressed as mean ± standard error of the mean (SEM).

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3. RESULTS

3.1. PIP treatment during IVM of porcine oocytes improves subsequent embryo development

To determine the optimal concentration of PIP, IVM medium was supplemented with 0, 50, 100, 200, or 400 μM PIP. The efficiency of oocyte maturation was determined by calculating the percentages of oocytes that exhibited PB extrusion after IVM for 44 h. Oocytes treated with 200 μM PIP (200 PIP) tended to have a higher development rate and the BL formation rate was notably increased. The BL formation rate at day 7 was 14% higher ($p < 0.05$) in the 200 PIP group (34.1 ± 4.0%) than in the control group (20.1 ± 2.4%) (Figure 1B). The total cell number per BL did not significantly differ between the groups (Figure 1D), but the percentage of apoptotic cells in BLs was 0.6% lower ($p < 0.05$) in the 200 PIP group (0.8 ± 0.1%) than in the control group (1.4 ± 0.2%) (Figure 1E). Therefore, 200 μM was selected as the optimal concentration of PIP, and subsequent experiments were conducted with the control and 200 PIP groups.

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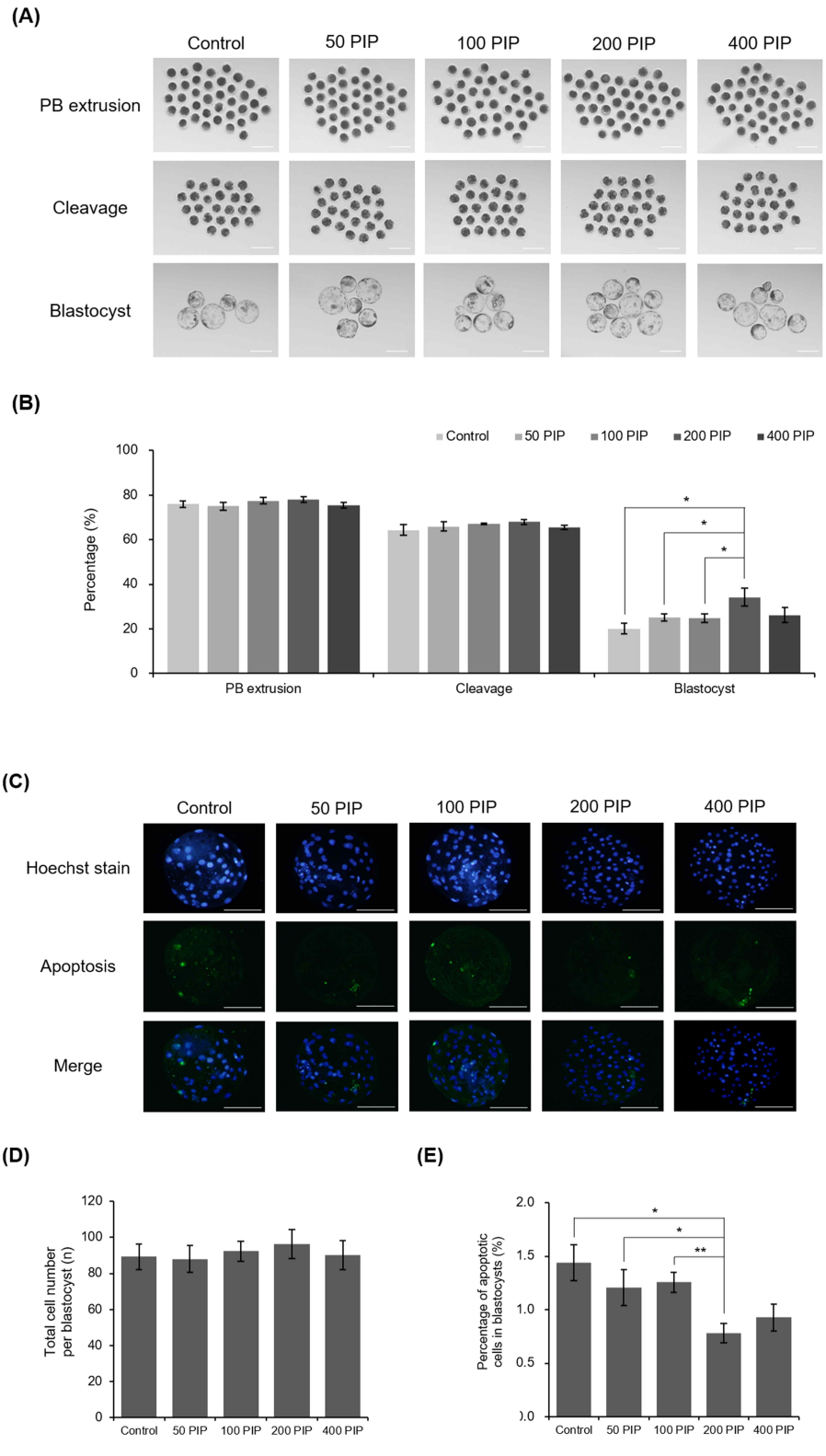

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Figure 1. Effect of treatment of porcine oocytes with various concentrations of PIP on subsequent embryo development *in vitro*. (A) PB extrusion, cleavage, and BL formation rates. (B) Total cell number per BL. (C) Percentage of apoptotic cells in BL. BLs were analyzed on day 7. Data are mean ± SEM from five independent experiments ($^{*}p < 0.05$, and $^{**}p < 0.01$).

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3.2. PIP treatment elicits antioxidant effects during IVM of porcine oocytes

We investigated the antioxidant effects of PIP treatment on porcine oocytes. DCFHDA and CMF_2HC were used to measure the levels of ROS and GSH at the MII stage (Figure 2A). The intracellular ROS level, as determined by the intensity of DCFHDA staining, was lower ($p < 0.05$) in the 200 PIP group (54.6 ± 1.3 pixels/oocytes) than in the control group (58.4 ± 1.4 pixels/oocytes) (Figure 2B). The intracellular GSH level, as determined by the intensity of CMF_2HC staining, was higher ($p < 0.05$) in the 200 PIP group (106.0 ± 2.0 pixels/oocytes) than in the control group (99.7 ± 2.5 pixels/oocytes) (Figure 2B). We next examined expression of antioxidant genes (nuclear factor erythroid 2-related factor 2 [*Nrf2*], catalase [*CAT*], heme oxygenase 1 [*HO-1*], superoxide dismutase 1 [*SOD1*], and superoxide dismutase 2 [*SOD2*]) by real-time RT-PCR at the MII stage. Expression of these genes was higher ($p < 0.05$) in the 200 PIP group than in the control group (Figure 2C).

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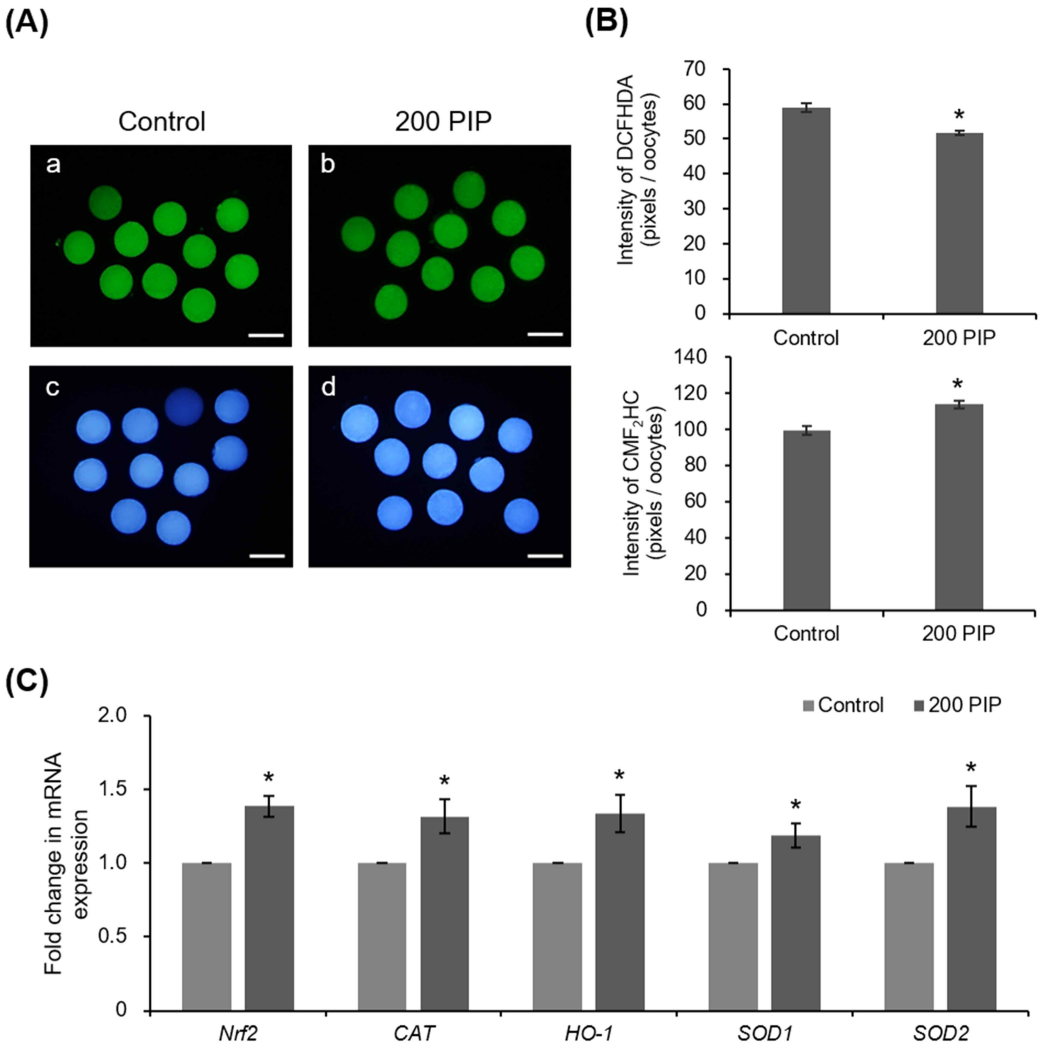

Figure 2. Antioxidant effect of PIP on porcine oocytes during IVM. (A) Representative images of oocytes stained with DCFHDA (green, a - b, ROS staining) and CMF_2HC (blue, c - d, GSH staining). Scale bar, 100 μm. (B) Quantification of the fluorescence intensities of DCFHDA and CMF_2HC. Oocytes were analyzed at the MII stage. The experiment was independently repeated six times with 10 oocytes per experiment. (C) Relative expression of antioxidant genes (*Nrf2*, *CAT*, *HO-1*, *SOD1*, and *SOD2*). Data are mean ± SEM from six independent experiments (* $p < 0.05$).

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3.3. PIP treatment prevents aberrant spindle organization and chromosome misalignment during IVM of porcine oocytes

To investigate nuclear maturation of porcine oocytes, we evaluated chromosome alignment and spindle organization. We classified oocyte morphology as normal or abnormal (Lenie, et al. 2008) (Figure 3A). The percentage of oocytes with normal spindle organization and chromosome alignment was higher ($p < 0.01$) in the 200 PIP group (88.4% ± 0.9%) than in the control group (82.2% ± 1.3%) (Figure 3B).

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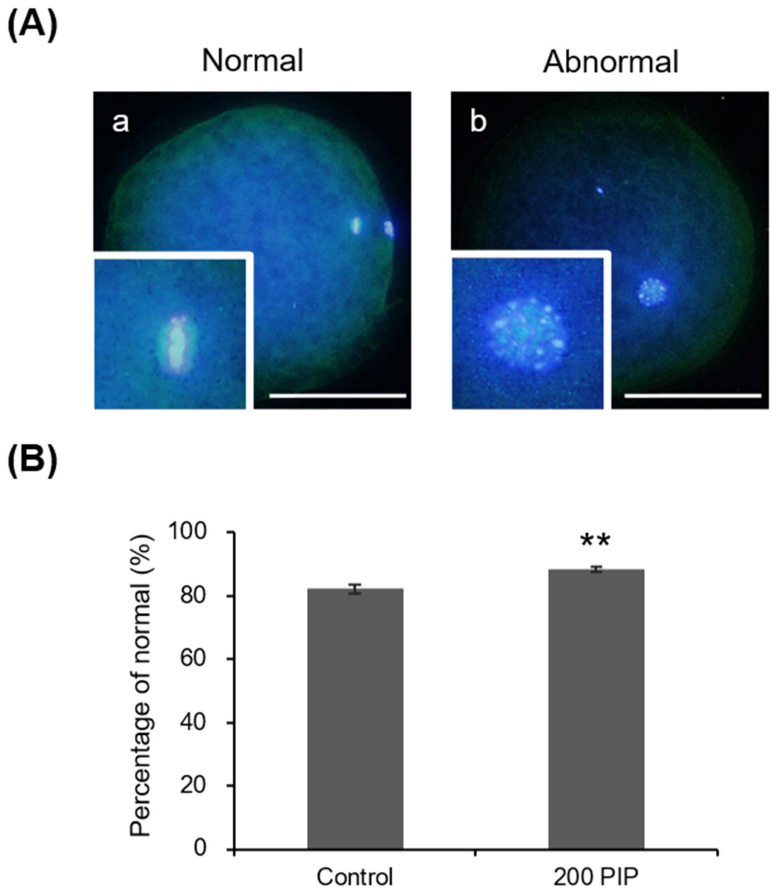

Figure 3. Effect of PIP on chromosome alignment and spindle organization in porcine oocytes *in vitro*. (A) Representative images of oocytes with normal and abnormal morphologies. Scale bar, 50 μm. (B) Percentage of oocytes with normal chromosome alignment and spindle morphology. Oocytes were analyzed at the MII stage. Data are mean ± SEM from six independent experiments (** $p < 0.01$).

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3.4. PIP treatment increases expression of a cytoplasmic maturation marker during IVM of porcine oocytes

We investigated cytoplasmic maturation of porcine oocytes, which is important for embryo development. In western blotting, phospho-p44/42 MAPK, the active form of this kinase, migrated as a doublet in lysates of matured porcine oocytes (Figure 4A). The ratio of phospho-p44/42 to p44/42 was higher ($p < 0.001$) in the 200 PIP group (1.5 ± 0.08) than in the control group (1.0 ± 0.0) (Figure 4B).

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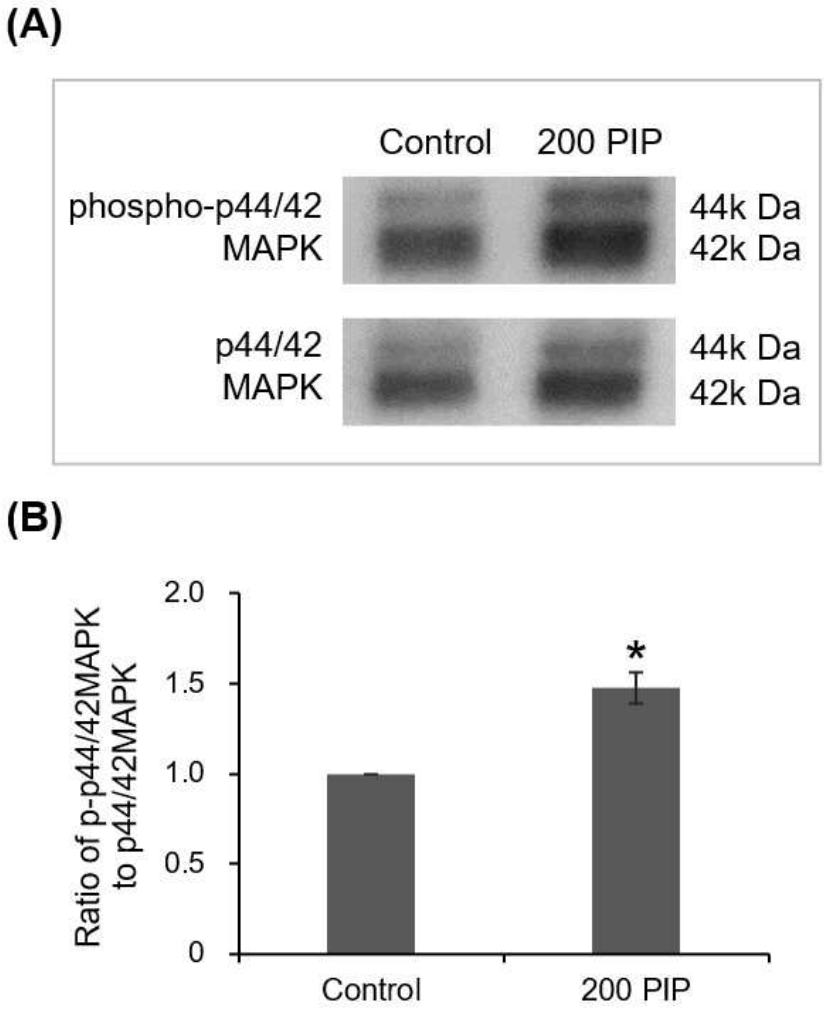

Figure 4. Effect of PIP on cytoplasmic maturation of porcine oocytes *in vitro*. (A) Western blot analysis of total and phosphorylated p44/42 MAPK. (B) Ratio of phospho-p44/42 MAPK to p44/42 MAPK. Oocytes were analyzed at the MII stage. Data are mean ± SEM from six independent experiments (*** $p < 0.001$).

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3.5. PIP treatment alters gene expression in embryos during IVM of porcine oocytes

To investigate the molecular mechanism underlying the improved quality of embryos that develop from PIP-treated porcine oocytes, expression of development-related (caudal type homeobox protein 2 [*CDX2*], Nanog homeobox [*NANOG*], POU domain, class 5, transcription factor 1 [*POU5F1*], and sex-determining region Y-box transcription factor 2 [*SOX2*]) and apoptosis-related (BCL2-like 1 [*BCL2L1*], baculoviral IAP repeat-containing 5 [*BIRC5*], Bcl-2 antagonist/killer [*BAK*], Fas cell surface death receptor [*FAS*], and cysteine-aspartic acid protease 3 [*CASP3*]) genes was analyzed by real-time RT-PCR in BLs at day 7. Expression of development-related genes (*CDX2*, *NANOG*, *POU5F1*, and *SOX2*) was higher ($p < 0.05$) in the 200 PIP group than in the control group, and expression of *POU5F1* was particularly higher ($p < 0.001$) in the former group than in the latter group. Expression of anti-apoptotic genes (*BCL2L1* and *BIRC5*) was higher ($p < 0.05$) in the 200 PIP group than in the control group, and expression of *BCL2L1* was particularly higher ($p < 0.01$) in the former group than in the latter group. Expression of pro-apoptotic genes (*BAK*, *FAS*, and *CASP3*) was lower ($p < 0.05$) in the 200 PIP group than in the control group.

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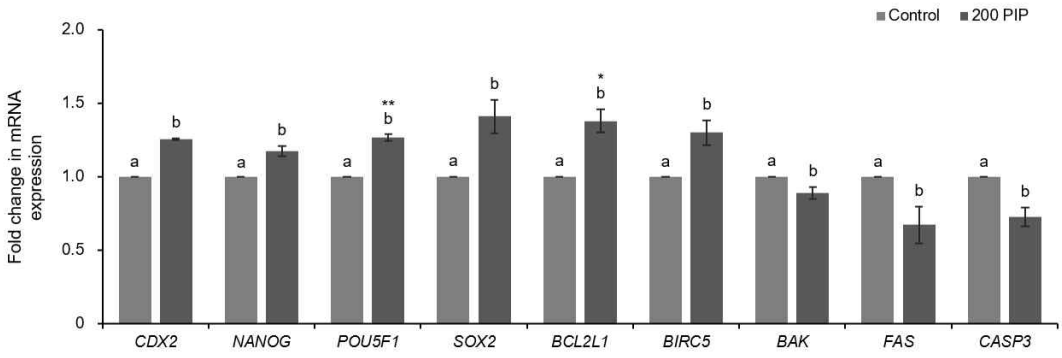

Figure 5. Effect of PIP treatment during IVM of porcine oocytes on gene expression in embryos derived from these oocytes. Relative expression of pluripotency-related (*CDX2*, *NANOG*, *POU5F1*, and *SOX2*), anti-apoptotic (*BCL2L1* and *BIRC5*), and pro-apoptotic (*BAK*, *FAS*, and *CASP3*) genes. Data are mean ± SEM from six independent experiments (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

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3.6. PIP treatment during IVM of porcine oocytes improves subsequent embryo development following SCNT

We evaluated the effect of PIP treatment of porcine oocytes on the developmental capacity and quality of BLs formed following SCNT. Oocytes that exhibited PB extrusion after IVM for 36 - 38 h were used. The developmental capacity of porcine oocytes was determined by calculating the fusion, cleavage, and BL formation rates. These rates were higher ($p < 0.05$) in the 200 PIP group than in the control group (Figure 6B). The quality of BLs was determined by calculating the total cell number per BL and the percentage of apoptotic cells in BLs. The total cell number per BL did not significantly differ between the groups, while the apoptosis rate was lower in the 200 PIP group (1.4% ± 0.2%) in the control group (2.4% ± 0.3%) (Figure 6E).

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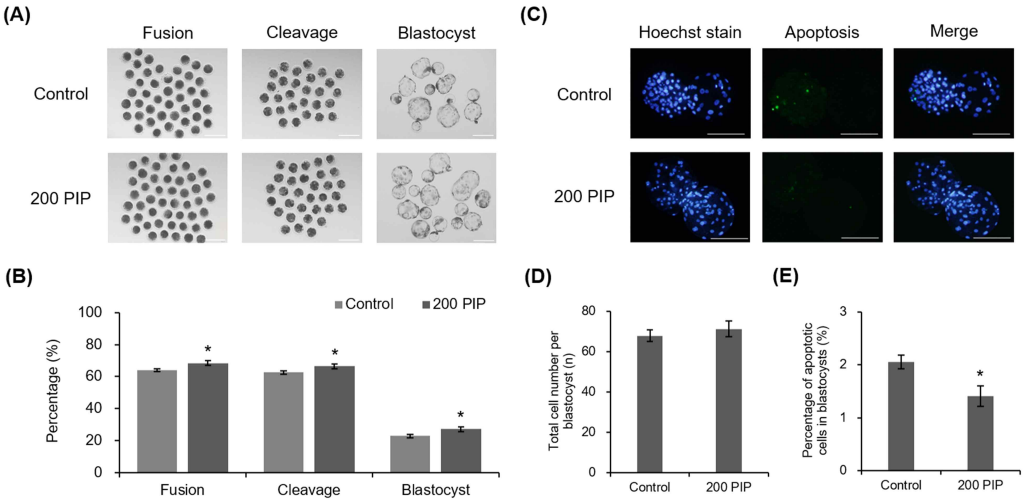

Figure 6. Effect of PIP treatment during IVM of porcine oocytes on BLs derived from these oocytes *via* SCNT. (A) Representative images of oocytes *via* SCNT. Scale bar, 100 μm. (B) Fusion, cleavage, and BL formation rates. (C) Blastocyst staining. Scale bar, 160 μm. (D) Total cell number per BL. (E) Percentage of apoptotic cells in BL. BLs were analyzed on day 7. Data are mean ± SEM from four independent experiments (* $p < 0.05$).

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4. DISCUSSION

The quality of oocytes critically affects subsequent embryo development (Keefe, Kumar and Kalmbach 2015; Krisher, et al. 2007). IVM is inherently less stable than *in vivo* maturation, leading to excessive ROS and oxidative stress (Cao, et al. 2022). Elevated ROS levels can have detrimental effects on oocytes, including induction of DNA fragmentation and apoptosis (Agarwal, et al. 2006; Morado, et al. 2009). To mitigate these detrimental effects during IVM, we investigated the antioxidant effects of PIP on porcine oocytes. The developmental rate tended to be higher in the 200 PIP group and the BL formation rate was notably increased (Figure 1A). Additionally, the percentage of apoptotic cells in BLs was significantly lower in the 200 PIP group (Figure 1C). BL formation is a critical indicator of the embryo development capacity (Deng, et al. 2020). Although apoptosis typically occurs during development and aging and can help to maintain antioxidant mechanisms (Elmore 2007), excessive apoptosis can negatively affect embryo development by inhibiting BL formation (Brison and Schultz 1997). Thus, BL formation and regulation of apoptosis are key factors in early embryo development. Based on these results, the 200 PIP group was further studied.

To assess whether PIP improves embryo development by eliciting antioxidant effects, we quantified intracellular ROS and GSH levels. The intracellular redox balance can be maintained through the equilibrium of ROS and GSH (Holmström and Finkel 2014). ROS play vital roles in cell signaling and homeostasis (Armand, et al. 2019), but excessive ROS can lead to mitochondrial dysfunction and cell death, ultimately impairing embryo development (Bedaiwy, et al. 2004; Hu, et al. 2001). Meanwhile, GSH protects oocytes against oxidative stress and is essential for cytoplasmic maturation

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(de Matos, et al. 1995; Luberda 2005). In the 200 PIP group, the intracellular ROS level was significantly decreased and the intracellular GSH level was significantly increased (Figure 2A, B). Consistently, expression of antioxidant genes was increased in the 200 PIP group (Figure 2C). Among these genes, *Nrf2* encodes a protein that plays a vital role in regulating antioxidant enzymes, including those encoded by *CAT*, *HO-1*, and *SOD* (Lee, et al. 2005; Li, et al. 2008). MAPK and phosphatidylinositol 3-kinase/protein kinase B are involved in nuclear translocation and activation of *Nrf2* (Lee, et al. 2015), and activated *Nrf2* regulates the antioxidative reaction by binding to the antioxidant response element (Cho, et al. 2008). Increased expression of *Nrf2* might signify enhancement of cellular defense mechanisms against oxidative stress (Dreger, et al. 2009). Additionally, elevated expression of antioxidant enzymes regulated by *Nrf2* may strengthen cellular protection (Dreger, et al. 2009). *CAT* may protect oocytes by metabolizing hydrogen peroxide and free radicals into non-reactive molecules (Fatehi, et al. 2005; Tiedge, et al. 1997), while *HO-1* regulates the immune response and maintain intracellular homeostasis (Bhang, et al. 2011; Katori, Busuttil and Kupiec-Weglinski 2002; Öllinger and Pratschke 2010). Moreover, *SOD* converts superoxide radicals into hydrogen peroxide and molecular oxygen (Fridovich 1986; McCord and Fridovich 1988). Our results regarding antioxidant enzymes are consistent with the previous findings that PIP activates *Nrf2* (Choi, et al. 2007), upregulates intracellular antioxidant enzymes (*CAT* and *SOD*), and regulates the *Nrf2/HO-1* antioxidative pathway (Duan, et al. 2022). These results demonstrate that PIP elicits antioxidant effects by activating the antioxidative pathway related to *Nrf2* and reducing oxidative stress in porcine oocytes.

Mitigation of oxidative stress by PIP during IVM may facilitate normal oocyte maturation. Oocyte maturation comprises nuclear and cytoplasmic maturation and is essential for subsequent development (Akar,

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Ahmad and Khalıd 2018). Normal chromosome alignment and spindle formation are important for oocyte maturation (Clift and Schuh 2015). Oxidative stress during IVM decreases the percentage of oocytes that reach the MII stage and results in spindle defects and chromosome misalignment (Alvarez, et al. 2015; Banan, et al. 2002; Han, et al. 2017; Wang, et al. 2017), suggesting that it may arrest embryo development. We evaluated nuclear maturation of oocytes by analyzing spindle formation and chromosome alignment. The percentage of oocytes with normal chromosome alignment and spindle formation was higher in the 200 PIP group than in the control group (Figure 3). Moreover, phosphorylated p44/42 MAPK activity was increased in the 200 PIP group (Figure 4). MAPK plays a role in regulation of meiosis (Inoue, et al. 1996) by activating the ERK signaling pathway and participating in the signal transduction pathway from extracellular signals to the nucleus (Michael, Wurtzel and Goldfinger 2016; Yuen, et al. 2012). This pathway activates ERK (Yuan, et al. 2021), and activated ERK1/2 enhances porcine oocyte meiotic maturation (Kim, et al. 2020). The MAPK-ERK1/2 pathway can regulate cellular activities including differentiation, proliferation, and apoptosis (Cargnello and Roux 2011; Roux and Blenis 2004). The finding that MAPK activity was increased in the 200 PIP group is consistent with the previous finding that antioxidant treatment activates the MAPK-ERK pathway (Chen, et al. 2017; Ruiz-Medina, et al. 2019). Our results indicate that PIP enhances nuclear and cytoplasmic maturation of oocytes by decreasing ROS levels at the MII stage.

To explore the molecular mechanisms by which PIP promotes embryo development, we examined changes in mRNA expression. When mammalian zygotes develop into BLs, they comprise two groups of cells: the trophectoderm, which differentiates into trophoblast cells and constructs the placenta (Cross 2005), and the inner cell mass, which consists of pluripotent

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cells and develops into the embryo (Bou, et al. 2017). To evaluate formation and maintenance of the trophectoderm, *CDX2* may be a marker (Niwa, et al. 2005; Yamanaka, et al. 2006) and was recently reported to be important for early porcine embryo development (Bou, et al. 2017). Meanwhile, *POU5F1*, *SOX2*, and *NANOG* encode essential pluripotency regulators for early embryo development in the inner cell mass (Cauffman, et al. 2004; Chambers, et al. 2003; Masui, et al. 2007). A recent study reported that antioxidant treatment not only influences development-related genes, including *CDX2*, *POU5F1*, *NANOG*, and *SOX2*, but also improves porcine embryo development (Pyeon, et al. 2021). Expression of these development-related genes was significantly upregulated in the 200 PIP group (Figure 5), consistent with our finding that embryo development was improved (Figure 1).

Considering that oxidative stress can cause apoptosis, we analyzed expression of apoptosis-related genes. Given that excessive apoptosis can impair BL maturation, potentially leading to early embryo death (Brison and Schultz 1997), assessment of apoptosis during embryo development is essential. In the main apoptosis pathway, mitochondria mediate activation of caspase cascades (Jeong and Seol 2008). Cytochrome c enters the cytoplasm and combines with caspase-9 to activate caspase-3 (Sinha, et al. 2013), and this process is regulated by BCL2 family proteins. The BCL2 family consists of both anti-apoptotic and pro-apoptotic proteins (Adams, et al. 2019). Anti-apoptotic gene expression is decreased by oxidative stress (Wang, et al. 2009) and increased by antioxidant treatment (Yoon, et al. 2021). *BCL2L1* and *BIRC5* encode anti-apoptotic regulators (Contis, et al. 2018; Reed 1997), while *BAK* encodes a mitochondrial pro-apoptotic regulator (Birkinshaw and Czabotar 2017). On the other hand, *FAS* encodes a protein that stimulates the initiator caspase-8, which propagates the apoptotic signal (Walczak and Krammer 2000). Caspases induce apoptosis by cleaving various substrates in

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the cytoplasm and nucleus (Degterev, Boyce and Yuan 2003) and ultimately provoke the main mediator of mammalian apoptosis, cysteine-aspartic acid protease 3 (*CASP3*) (Landgraeber, et al. 2008). PIP treatment reduces cytochrome c release and regulates caspase-3 and -9 activated by 6-OHDA in a rat model (Shrivastava, et al. 2013). Furthermore, PIP reduces apoptosis by maintaining a balance between pro-apoptotic and anti-apoptotic proteins (Kaushik, et al. 2021). In the 200 PIP group, anti-apoptotic genes were upregulated and pro-apoptotic genes were downregulated (Figure 5), supporting the idea that PIP maintains a balance between pro-apoptotic and anti-apoptotic proteins. This is also consistent with our finding that the level of apoptosis was reduced in the 200 PIP group (Figure 1).

Embryos generated by PA can quickly confirm experimental effects. We treated porcine oocytes with various concentrations of PIP to determine the optimal concentration (Figure 1). Thereafter, we investigated the influence of PIP on the level of oxidative stress in porcine oocytes, oocyte maturation, and molecular mechanisms in embryos derived from these oocytes (Figures 2 - 5). Based on these results, we finally investigated the influence of PIP on embryos generated by SCNT (Figure 6). SCNT can potentially harm oocytes due to transfer of the donor somatic cell nucleus into the cytoplasm and subsequent fusion (Gouveia, et al. 2020). Antioxidant treatment effectively protects the cytoplasm and improves development of SCNT embryos (Mitalipov, et al. 2007). Consistently, development of SCNT embryos was significantly improved in the 200 PIP group (Figure 6A). We also assessed the quality of BLs by quantifying the total cell number per BL and the percentage of apoptotic cells in BLs (Knijn, et al. 2003). The total cell number per BL did not significantly differ between the groups and the apoptosis rate was lower in the 200 PIP group than in the control group (Figure 6B). In summary, PIP treatment positively influenced SCNT embryos

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by protecting their cytoplasm and enhancing oocyte maturation. This is consistent with the finding that embryo development following PA was improved in the 200 PIP group (Figure 1).

Our study determined the effect of treatment of porcine oocytes with PIP on subsequent embryo development following PA and SCNT. Treatment of porcine oocytes with PIP improved subsequent development of embryos generated by PA and regulated intracellular ROS and GSH levels. Furthermore, it enhanced subsequent embryo development by promoting nuclear and cytoplasmic maturation of porcine oocytes. These results suggest that PIP may improve the outcomes of ARTs by reducing intracellular ROS levels.

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피페린이 산화 스트레스를 감소시켜 체외 성숙 동안 돼지 난모세포의 품질에 미치는 영향

임은서

제주대학교 대학원 분자생명공학전공

빛과 고온으로 인한 산화 스트레스는 체외 성숙 중에 발생하여 생체 내에서 얻은 배아에 비해 품질이 낮은 배아를 생성한다. 이 문제를 극복하기 위해 우리는 돼지 난모세포의 성숙 과정에서 피페린 처리가 체외에서 후속 배아 발달에 미치는 영향을 조사했다. 돼지 난모세포는 0, 50, 100, 200 또는 400 μM 피페린이 보충된 체외 성숙 배지에서 배양되었다. 단위생식 활성화 후, 200 μM 피페린 처리 난모세포(200 PIP)를 사용하여 배반포 형성 속도가 상당히 높았고 배반포에서 세포사멸 세포의 비율이 상당히 낮았다. 200 PIP 그룹에서는 중기 II 단계의 활성 산소종 수준이 감소하고, 글루타티온 수준이 증가하고 항산화 유전자(*Nrf2*, *CAT*, *HO-1*, *SOD1* 및 *SOD2*)의 발현이 증가하였다. 일관되게 염색체 오정렬과 비정상적인 방추 조직이 완화되었고 인산화된 p44/42 MAPK 활성이 200 PIP 그룹에서 증가하였다. 발달 관련(*CDX2*, *NANOG*, *POU5F1* 및 *SOX2*), 항-아폽토시스(*BCL2L1* 및 *BIRC5*) 및 프로-아폽토시스(*BAK*, *FAS* 및 *CASP3*) 유전자의 발현은 200 PIP 그룹에서 조절되었다. 체세포 핵 이식 후 배아 발달은 200 PIP 그룹에서 개선되었다. 이러한 발견은 피페린이 체외 성숙 중에 필연적으로 발생하는 산화 스트레스를 감소시켜 돼지 난모세포의 품질을 향상시킨다는 것을 시사한다. 돼지 난모세포에 대한 심층적인 기계적 연구를 통해 보조 생식 기술의 효율성이 향상될 것이다.

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