A Dissertation for the Degree of Master of Science

Protective effect of protodioscin against H_2O_2-induced oxidative stress during *in vitro* maturation of porcine oocytes

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GRADUATE SCHOOL

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Protective effect of protodioscin against H_2O_2-induced oxidative stress during *in vitro* maturation of porcine oocytes

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CONTENTS……...……i

LIST OF FIGURES……iii

LIST OF TABLES…….……...iv

ABSTRACT……...1

1. INTRODUCTION……2

2. MATERIALS AND METHODS……..4

2.1. Chemicals and reagents……...4

2.2. *In vitro* maturation of porcine oocytes …….…..4

2.3. Parthenogenetic activation (PA) and embryo culture ....…….....4

2.4. Measurement of intracellular ROS and GSH levels ..……....5

2.5. Immunofluorescence……...6

2.6. Terminal deoxynucleotidyl transferase dUTP nick-end labeling and Hoechst staining……...6

2.7. mRNA extraction and complementary DNA synthesis…….....7

2.8. Real-time RT-PCR …….....7

2.9. Western blot analysis……9

2.10. Statistical analysis……..…….....9

3. RESULTS……11

3.1. PD enhances *in vitro* development of porcine oocytes exposed to oxidative stress……...…….11

3.2. PD protects porcine oocytes against oxidative stress ……..13

3.3. PD prevents aberrant spindle formation and abnormal chromosomal alignment in porcine oocytes exposed to oxidative stress…….……..15

3.4. PD increases expression of a cytoplasmic maturation marker in porcine oocytes exposed to oxidative stress……..……16

3.5. PD improves the quality of blastocysts derived from porcine oocytes exposed to oxidative stress *in vitro* …….17

3.6. PD alters expression of development-related genes in porcine oocytes exposed to oxidative stress ……...……..18

4. DISCUSSION……...19

REFERENCES……23

ABSTRACT IN KOREAN……27

ACKNOWLEDGEMENT……..28

LIST OF FIGURES

Figure. 1. Antioxidant effect of PD on porcine oocytes *in vitro*. A: Epifluorescence images of oocytes stained with DCFHDA (green) and CellTracker^{TM} Blue CMF_2HC (blue), and the fluorescence intensities of intracellular ROS and GSH staining. a and a': normal group; b and b': control group; and c and c': 10PD group. a, b, and c: ROS staining; a', b', and c': GSH staining. B: Relative expression of the antioxidant genes *SOD1, SOD2, Nrf2,* and *HO-1*. Data were derived from 3-8 independent replicates per group. Data are the means ± SEM ($^{a-b}p < 0.05$). Scale bar = 120 μm.

Figure. 2. Effect of PD on meiotic spindle morphology in porcine oocytes *in vitro*. Normal and abnormal chromosomal alignment and meiotic spindle formation in oocytes and percentage of oocytes in which the morphologies of chromosomes and the meiotic spindle were normal. Data were derived from 3-4 independent replicates per group. Data are the means ± SEM ($^{a-b}p < 0.05$). Scale bar = 50 μm.

Figure. 3. Effect of PD treatment of porcine oocytes *in vitro* on MAPK activity. Data were normalized against the levels in the control group and were derived from 6-7 independent replicates per group. Data are the means ± SEM ($^{a-c}p < 0.05$).

Figure. 4. Effect of PD treatment of porcine oocytes *in vitro* on subsequent embryo quality after PA. A: Blastocyst staining. B: Total cell number per blastocyst. C: Percentage of apoptotic cells in blastocysts. Data were derived from 7-8 independent replicates per group. Data are the means ± SEM ($^{a-b}p < 0.05$).

Figure. 5. Effect of PD treatment of porcine oocytes *in vitro* on expression of development-related genes. Data were derived from 3-4 independent replicates per group. Data are the means ± SEM ($^{a-b}p < 0.05$).

LIST OF TABLES

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

Table 2. Effect of PD treatment of porcine oocytes *in vitro* on subsequent embryo development

ABSTRACT

The present study investigated whether protodioscin (PD), a steroidal saponin mainly found in rhizome of Dioscorea species, alleviates oxidative stress-induced damage of porcine oocytes during *in vitro* maturation. Oocytes were treated with different concentrations of PD (0, 1, 10, 100, and 200 μM) in the presence of 200 μM H_2O_2. Developmental competence was significantly poorer in the 0 μM PD-treated (control) group than in the non-treated (normal) and 10 μM PD-treated (10PD) groups. Although the reactive oxygen species level did not significantly differ between these three groups, the glutathione level and mRNA expression of antioxidant genes (*SOD1*, *SOD2*, *Nrf2*, and *HO-1*) were significantly higher in the normal and 10PD groups than in the control group. In addition, the percentage of oocytes with defective spindle and abnormal chromosomal alignment was significantly lower and the ratio of phosphorylated p44/42 to total p44/42 was significantly higher in the normal and 10PD groups than in the control group. The total cell number per blastocyst was significantly higher in the 10PD group than in the control group. The percentage of apoptotic cells in blastocysts was highest in the control group; however, the difference was not significant. mRNA expression of development-related genes (*POU5F1*, *CDX2*, and *NANOG*) was consistently increased by addition of PD. These results indicate that PD effectively improves the developmental competence and quality of blastocysts by protecting porcine oocytes against oxidative stress.

Key words: ROS, Oxidative stress, Antioxidant, Protodioscin, *In vitro* maturation

1. INTRODUCTION

In vitro embryo production is an important tool in agriculture, biomedical research (Yao et al., 2019), and assisted reproductive technology (Hansen, 2020; Soto-Heras & Paramio, 2020). Similar to the *in vivo* system, *in vitro* embryo production comprises three major consecutive steps: oocyte maturation, fertilization, and embryo culture. During oocyte development, unstable metabolites of oxygen known as reactive oxygen species (ROS) are generated as mitochondria produce energy via oxidative phosphorylation using oxygen or comes from their external environment (Al-Gubory, Fowler, & Garrel, 2010; Guerin, El Mouatassim, & Menezo, 2001). An excessive level of ROS, i.e., an imbalance between endogenous antioxidant defense and ROS, leads to DNA fragmentation and apoptosis, and thereby decreases the developmental capacity of oocytes and blastocyst quality (Adeoye, Olawumi, Opeyemi, & Christiania, 2018; W. Jiang et al., 2020). Thus, protection of oocytes against oxidative stress is important to improve the efficiency of *in vitro* embryo production (Paramio & Izquierdo, 2014; Soto-Heras & Paramio, 2020). Addition of antioxidants to media is one of the most fundamental and easiest strategies to improve embryo quality during *in vitro* culture (Jeong et al., 2006; Takahashi et al., 1993).

Protodioscin (PD), a furostanol saponin obtained from the rhizome of Dioscorea species, has a wide array of biological activities such as anticancer (Hu and Yao, 2002), anti-inflammatory (Santana et al., 2009), and antioxidant effects. PD reduces oxidative stress, as demonstrated by increases of superoxide dismutase (SOD) and glutathione (GSH) peroxidase activities, and decreases of ROS and malondialdehyde levels in neural cells. In addition, inhibition of oxidative stress and apoptosis was observed together with increased expression of heat shock proteins in the presence of PD (Shu & Zhang, 2019). Although several studies reported that PD has beneficial effects on oxidative stress-induced damage

and transcriptional regulation in neural cells, very little is known about the effect of PD in oocytes.

In the present study, we hypothesized that PD protects porcine oocytes against H_2O_2-induced oxidative stress during *in vitro* maturation (IVM) and investigated the quality of oocytes and embryos obtained by parthenogenetic activation (PA). Furthermore, we evaluated the ROS level, GSH activity, and mRNA expression of endogenous antioxidant and development-related genes. Cytoplasmic and nuclear maturation was also assessed to better understand the beneficial effects of PD. These findings may help to develop embryo production technology by facilitating further research of the mechanism via which PD inhibits oxidative stress in germ cells.

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. *In vitro* maturation 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. Various concentrations [0 (control group), 1, 10, 100, and 200 μM] of PD were added together with 200 μM H_2O_2. For the normal group, neither PD nor H_2O_2 was added. Each experiment was independently repeated six times, with 50–60 oocytes per experiment. All data are presented as the means ± SEM.

2.3. Parthenogenetic activation (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 (*in vitro* culture (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 with IVC medium and cultured for 7 days in the same medium at 38.8°C in a humidified atmosphere of 5% CO_2 and 95% air. On day 5, half the medium was removed and replaced with PZM-5 containing 10% (v/v) fetal bovine serum. On day 7, blastocysts 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 lysed and snap-frozen in liquid nitrogen and stored at -80°C, depending on the experiment.

2.4. 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 (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. Each experiment was independently repeated 6–7 times, with 20–30 oocytes per experiment.

2.5. Immunofluorescence

Meiotic spindles and nuclei of oocytes were visualized after maturation. Cumulus cells were removed from porcine COCs matured for 44 hr 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 PD 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.

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

On day 7 after PA, blastocysts were fixed overnight at 4°C with 4.0% (w/v) paraformaldehyde prepared in PBS, washed 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. Thereafter, nuclei were stained with Hoechst 33342 (1 μg/mL) for 30 min, and stained blastocysts were washed with PBS containing 0.1% BSA. Washed blastocysts were mounted on glass slides and examined under an inverted Olympus IX-71 fluorescence microscope. The experiment was independently repeated 7–8 times, and at least 10–20 blastocysts were examined per group.

2.7. 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.

2.8. 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 2 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 the housekeeping gene *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)
β-actin	AY550069.1	F: AGATCATGTTCGAGACCTTC R: GTCAGGATCTTCATGAGGTAGT	49	220
SOD1	GU944822.1	F: GTGTTAGTAACGGGAACCAT R: GGATTCAGGATTGAAGTGAG	54	120
SOD2	NM_214127.2	F: AGACCTGATTACCTGAAAGC R: CTTGATGTACTCGGTGTGAG	54	110
Nrf2	Gu991000.1	F: CTATGGAGACACACTGCTTG R: ACAGGCTGTGTTTTAGGACT	54	99
HO-1	NM001004027.1	F: ACCCAGGACACTAAGGACCA R: CGGTTGCATTCACAGGGTTG	54	227
POU5F1	NM_001113060	F: AGTGAGAGGCAACCTGGAGA R: TCGTTGCGAATAGTCACTGC	54	166
CDX2	AM778830	F: AGCCAAGTGAAAACCAGGAC R: TGCGGTTCTGAAACCAGATT	48	178
NANOG	DQ447201	F: TTCCTTCCTCCATGGATCTG R: ATCTGCTGGAGGCTGAGGTA	53	214

*F, forward; R, reverse.

2.9. 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 on X-ray films using the chemiluminescent reagent luminol (Invitrogen) in a dark room. The experiment was independently repeated three times.

2.10. Statistical analysis

The general linear model procedure within the Statistical Analysis System (SAS User's Guide, 1985, Statistical Analysis System Inc., Cary, NC, USA) was used to analyze data from all experiments. The paired Tukey's multiple range test was used to determine

significant differences. p values less than 0.05 were defined as statistically significant.

3. RESULTS

3.1. PD enhances *in vitro* development of porcine oocytes exposed to oxidative stress

To determine the optimal concentration of PD, porcine oocytes were matured for 44 hr with 0, 1, 10, 100, and 200 μM PD (control, 1PD, 10PD, 100PD, and 200PD groups, respectively) in the presence of 200 μM H_2O_2. Oocytes in the normal group were matured in IVM medium without any supplements. Following PA, the percentage of cleaved oocytes on day 2 did not significantly differ between the groups (normal, 80.1 ± 2.5%; control, 78.6 ± 2.3%; 1PD, 74.8 ± 4.0%; 10PD, 78.4 ± 4.0%; 100PD, 80.7 ± 3.6%; and 200PD, 81.9 ± 2.6%). However, the percentage of oocytes that reached the blastocyst stage on day 7 was significantly higher in the normal and 10PD groups than in the control and 1PD groups, but did not significantly differ between these four groups and the 100PD and 200PD groups (normal, 38.9 ± 1.4%; control, 30.5 ± 3.3%; 1PD, 30.8 ± 3.4%; 10PD, 41.8 ± 2.9%; 100PD, 35.8 ± 4.7%; and 200PD, 38.5 ± 3.8%; Table 1). Therefore, the normal, control, and 10PD groups were compared in subsequent experiments.

Table 2. Effect of PD treatment of porcine oocytes *in vitro* on subsequent embryo development

Treatment group	H_2O_2 Concentration (μM)	PD concentration (μM)	No. of germinal vesicle oocytes	No. (%) of		
				Surviving Oocytes[1]	cleaved oocytes on Day 2[2]	Blastocysts on Day 7[3]
Normal	0	0	300	282 (94.0 ± 1.5)	226 (80.1 ± 2.5)	88 (38.9 ± 1.4)[b]
Control	200	0	300	280 (93.3 ± 1.6)	220 (78.6 ± 2.3)	67 (30.5 ± 3.3)[a]

1PD	200	1	300	282 (94.0 ± 1.3)	211 (74.8 ± 4.0)	65 (30.8 ± 3.4)[a]
10PD	200	10	300	287 (95.7 ± 1.4)	225 (78.4 ± 4.0)	94 (41.8 ± 2.9)[b]
100PD	200	100	300	280 (93.3 ± 1.3)	226 (80.7 ± 3.6)	81 (35.8 ± 4.7)[ab]
200PD	200	200	300	282 (94.0 ± 1.5)	231 (81.9 ± 2.6)	89 (38.5 ± 3.8)[ab]

[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-b]$p < 0.05$). PD, protodioscin.

3.2. PD protects porcine oocytes against oxidative stress

The effects of PD on the ROS and GSH levels were assessed by staining oocytes with dichlorohydrofluorescein diacetate (DCFHDA) and CellTracker^{TM} Blue 4-chloromethyl-6,8-difluoro-7-hydroxy-coumarin (CMF_2HC), respectively (Figure 1A). The ROS level did not significantly differ between the three groups. The GSH level was significantly higher ($p < 0.05$) in the normal and 10PD groups than in the control group.

Expression of the antioxidant genes *SOD1*, *SOD2*, nuclear factor erythroid 2-related factor 2 (*Nrf2*), and *HO-1* was analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR) (Figure 1B). mRNA expression of *SOD1* was significantly higher ($p < 0.05$) in the normal group than in the control group, and was substantially higher in the 10PD group than in the control group; however, this difference was not significant. mRNA expression of *SOD2* was significantly higher ($p < 0.05$) in the 10PD than in the normal and control groups, but did not significantly differ between the latter two groups. mRNA expression of *Nrf2* was significantly higher in the normal and 10PD groups than in the control group. The mRNA expression pattern of *HO-1* was similar to that of *Nrf2*.

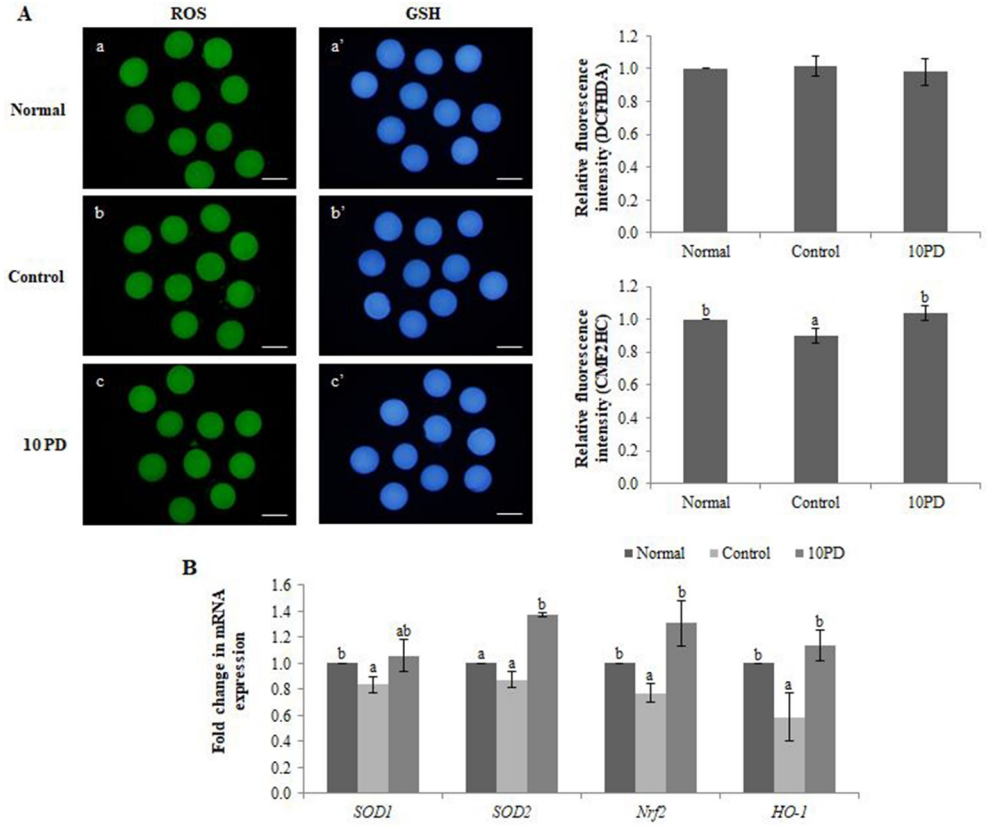

Figure. 1. Antioxidant effect of PD on porcine oocytes *in vitro*. A: Epifluorescence images of oocytes stained with DCFHDA (green) and CellTracker™ Blue CMF_2HC (blue), and the fluorescence intensities of intracellular ROS and GSH staining. a and a': normal group; b and b': control group; and c and c': 10PD group. a, b, and c: ROS staining; a', b', and c': GSH staining. B: Relative expression of the antioxidant genes *SOD1, SOD2, Nrf2,* and *HO-1*. Data were derived from 3-8 independent replicates per group. Data are the means ± SEM ($^{a-b}p < 0.05$). Scale bar = 120 μm.

3.3. PD prevents aberrant spindle formation and abnormal chromosomal alignment in porcine oocytes exposed to oxidative stress

The percentage of oocytes with a normal meiotic spindle and normal chromosomal alignment was significantly higher in the normal ($p < 0.01$) and 10PD ($p < 0.05$) groups than in the control group, and was similar in the normal and 10PD groups (normal, 80.5 ± 3.1%; control, 56.8 ± 6.4%; and 10PD, 78.0 ± 4.1%; Figure 2).

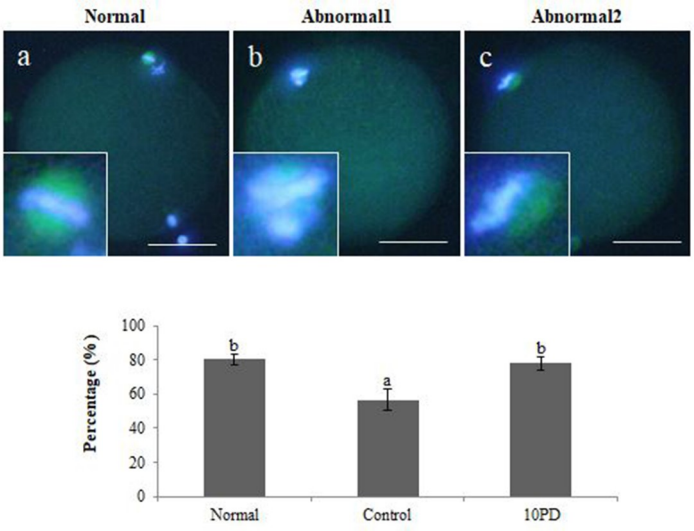

Figure. 2. Effect of PD on meiotic spindle morphology in porcine oocytes *in vitro*. Normal and abnormal chromosomal alignment and meiotic spindle formation in oocytes and percentage of oocytes in which the morphologies of chromosomes and the meiotic spindle were normal. Data were derived from 3-4 independent replicates per group. Data are the means ± SEM ($^{a-b}p < 0.05$). Scale bar = 50 μm.

3.4. PD increases expression of a cytoplasmic maturation marker in porcine oocytes exposed to oxidative stress

Several studies have suggested that mitogen-activated protein kinase (MAPK) phosphorylation is an important marker to evaluate cytoplasmic maturation. Therefore, we investigated whether PD improves porcine oocyte maturation via the MAPK signal transduction pathway. Lysates from the normal, control, and 10PD groups were immunoblotted with an anti-phosphorylated MAPK antibody and subsequently re-probed with an anti-MAPK antibody to normalize the densitometric results. MAPK migrates as a doublet at 44 and 42 kDa, representing p44/42 MAPK (ERK1/2). The ratio of phosphorylated MAPK (phospho-p44/42 MAPK), which is the active form, to total MAPK was significantly lower ($p < 0.05$) in the control group than in the normal and 10PD groups and was significantly higher ($p < 0.05$) in the 10PD group than in the normal group (normal, 1.0 ± 0.0; control, 0.8 ± 0.1; and 10PD, 1.2 ± 0.1; Figure 3).

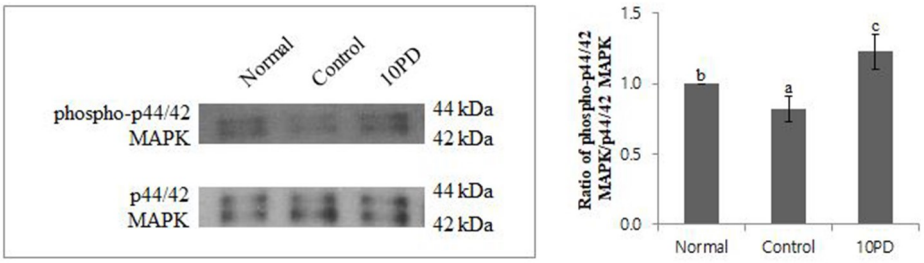

Figure. 3. Effect of PD treatment of porcine oocytes *in vitro* on MAPK activity. Data were normalized against the levels in the control group and were derived from 6-7 independent replicates per group. Data are the means ± SEM ($^{a-c}p < 0.05$).

3.5. PD improves the quality of blastocysts derived from porcine oocytes exposed to oxidative stress *in vitro*

To investigate whether PD treatment during IVM of porcine oocytes influences subsequent embryo development and quality, the total cell number and genomic DNA fragmentation in blastocysts were assessed (Figure 4A). The total cell number per blastocyst was significantly higher ($p < 0.05$) in the 10PD group than in the control group, and was slightly lower in the control group than in the normal group; however, this difference was not significant (normal, 78.0 ± 5.0; control, 73.2 ± 4.1; and 10PD, 85.0 ± 2.9; Figure 4B). The percentage of apoptotic cells in blastocysts determined by assessment of genomic DNA fragmentation was higher in the control group than in the normal and 10PD groups; however, this difference was not significant (normal, 2.6 ± 0.6%; control, 2.7 ± 0.3%; and 10PD, 1.8 ± 0.4%; Figure 4C).

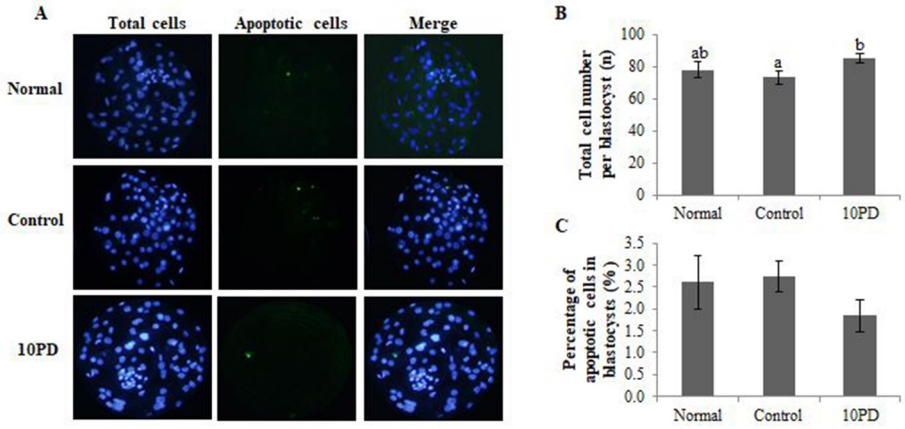

Figure. 4. Effect of PD treatment of porcine oocytes *in vitro* on subsequent embryo quality after PA. A: Blastocyst staining. B: Total cell number per blastocyst. C: Percentage of apoptotic cells in blastocysts. Data were derived from 7-8 independent replicates per group. Data are the means ± SEM ($^{a-b}p < 0.05$).

3.6. PD alters expression of development-related genes in porcine oocytes exposed to oxidative stress

Expression of the development-related genes *POU5F1*, *CDX2*, and *NANOG* at the blastocyst stage was analyzed by real-time RT-PCR (Figure 5). mRNA expression of *POU5F1* was slightly decreased by addition of H_2O_2 and increased by supplementation of PD, but did not significantly differ between the three groups. mRNA expression of *CDX2* was significantly higher ($p < 0.05$) in the 10PD group than in the normal and control groups, and was similar in the latter two groups. mRNA expression of *NANOG* was slightly lower in the control group than in the normal group, and was significantly higher ($p < 0.05$) in the 10PD group than in the control group.

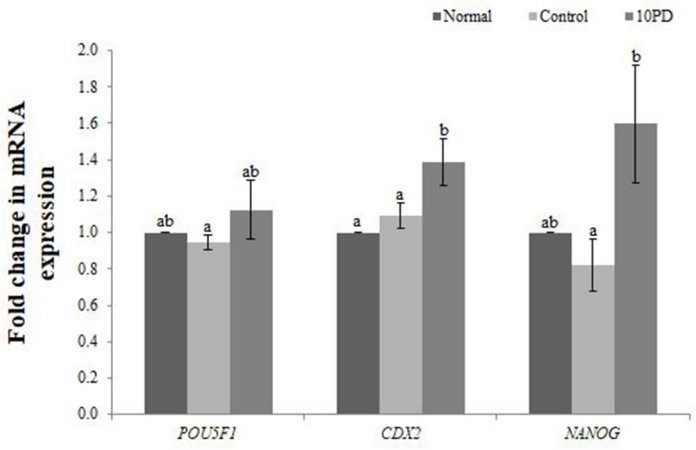

Figure. 5. Effect of PD treatment of porcine oocytes *in vitro* on expression of development-related genes. Data were derived from 3-4 independent replicates per group. Data are the means ± SEM ($^{a–b}p < 0.05$).

4. DISSCUSTION

Oxidative stress caused by ROS is an important cause of apoptosis, inhibition of oocyte maturation and early embryonic development (Agarwal, Gupta, & Sharma, 2005; W. J. Jiang et al., 2020). Several studies have shown that PD elicits antioxidant effects and reduces apoptosis caused by oxidative stress in neural cells (Shu & Zhang, 2019; Song, Fajol, Chen, Ren, & Shi, 2018). This study investigated the effects of PD on H_2O_2-induced oxidative stress in porcine oocytes. In the present study, we showed that addition of H_2O_2 during IVM significantly diminished the developmental capacity of porcine oocytes. However, supplementation of 10 μM PD significantly improved oocyte quality, which is impaired by H_2O_2 in a concentration-dependent manner, and consequently enhanced embryo development as reflected by the percentage of oocytes that reached the blastocyst stage (Table 1). Blastocyst formation is a critical indicator of the efficiency of embryo development and culture conditions (Deng et al., 2020; Watson, Natale, & Barcroft, 2004). Although the percentages of surviving oocytes at metaphase of the second meiotic division (MII) stage and cleaved oocytes did not significantly differ between the three groups, the percentage of oocytes that reached the blastocyst stage was significantly higher in the 10PD and normal groups than in the control group.

To find out whether changes in MII oocytes matured in an environment inducing oxidative stress affect subsequent embryo development, alterations in the ROS and GSH levels and spindle morphology of MII oocytes were investigated. Addition of PD to IVM medium containing H_2O_2 did not affect the ROS level in oocytes at the MII stage in comparison with the normal and control groups. However, the GSH level was significantly lower in the control group than in the normal group, and was slightly higher in the 10PD group than in the normal group. This suggests that the beneficial effect of PD on porcine oocytes is

attributable to an increase in endogenous antioxidants rather than a decrease in the ROS content. Similarly, mRNA expression of antioxidant genes (*SOD1*, *SOD2*, *Nrf2*, and *HO-1*) was consistently lower in the control group than in the normal and 10PD groups. This demonstrates that H_2O_2 remarkably decreases transcription of antioxidant genes and PD alleviates the effects of H_2O_2 and upregulates relative mRNA expression of these genes.

During meiotic maturation, formation of the spindle is very important for alignment of chromosomes, which is directly related to separation of chromosomes and normal development of embryos in meiosis, and failure of this process results in genetic disorders and aneuploid embryos (Huang et al., 2011; Yin, Sun, Schatten, & Sun, 2008). Several studies have shown that oxidative stress affects microtubule assembly in interphase cells, suggesting that ROS may affect spindle formation (Banan et al., 2002). For example, a delay of spindle formation was observed in a study using HeLa cells exposed to oxidative stress. Moreover, the appearance of misaligned chromosomes and multipolar spindles in metaphase is substantially increased in the presence of H_2O_2 (Wang et al., 2017). Likewise, in our study, the percentage of oocytes with normal spindle morphology was remarkably lower in the control group than in the normal and 10PD groups. Consistently, an aberrant configuration of chromosomes was observed more often in the control group than in the other groups. These results demonstrate that addition of H_2O_2 negatively affects spindle formation and leads to abnormal chromosomal alignment. However, PD attenuates the negative effects of H_2O_2 and promotes normal development of oocytes and embryos in meiosis.

PD treatment also considerably increased the phosphorylated MAPK level. MAPK plays crucial roles in regulation of oocyte maturation along with MPF, which is a complex of cyclin B and Cdc2. MAPK plays a vital role in early embryo development processes, such as initiation of the first meiotic division in GV stage, promotion of nuclear maturation, and oocyte maintenance at the MII stage (Zhao et al., 2020). Several studies also suggested that

phosphorylation of MAPK is an important marker to evaluate cytoplasmic maturation along with cyclin B2 levels (Sun, Lai, Bonk, Prather, & Schatten, 2001). In our study, the increased level of phosphorylated MAPK suggests that PD enhances MAPK activity in H_2O_2-treated oocytes.

The percentage of blastocysts obtained by PA and the average total cell number per blastocyst were higher in the 10PD group than in the control group. The total cell number per blastocyst indicates the quality of blastocysts (Knijn et al., 2003). It is a standard criterion for evaluating the quality of embryos and indicates how well embryos are developed. By contrast, increased apoptosis is an important indicator of inadequate *in vitro* conditions for oocytes (Kim et al., 2008). Apoptosis is a process of programmed cell death that occurs regularly to ensure a homeostatic balance between the rates of cell formation and cell death, and involves many genes. However, excessive apoptosis can induce degeneration of oocytes and death of early embryos, and also affect normal blastocyst formation (Chen et al., 2020). In this study, the average percentage of apoptotic cells in blastocysts was lower in the 10PD group than in the normal and control groups; however, this difference was not significant.

To further understand the effect of PD on development of embryos, we assessed expression of development-related genes. POU5F1 and NANOG play important roles in maintaining the pluripotency of embryonic stem cells and promoting cell proliferation (Boyer et al., 2005). Knockout of POU5F1 and NANOG inhibits blastocyst development (Simmet et al., 2018). Similarly, CDX2 is essential for viability and proliferation of blastocyst cells (Bou et al., 2017). Expression of *POU5F1*, *CDX2*, and *NANOG* was consistently higher in the 10PD group than in the normal and control groups. This demonstrates that the improvement of early embryonic development by PD is closely correlated with upregulation of these genes.

In conclusion, our data indicate that H_2O_2 negatively affects the development of oocytes and reduces the quality of embryos and blastocysts derived from these oocytes, while supplementation of PD improves the developmental rate and enhances the quality of oocytes, and increases expression of antioxidant and development-related genes. Our results also demonstrate that PD protects porcine oocytes against H_2O_2-induced oxidative stress by inducing production of several antioxidant enzymes, including SOD1, SOD2, Nrf2, and HO-1, and further promotes normal early embryo development by supporting meiosis, especially spindle formation, to occur at an appropriate time and in an appropriate manner. PD only subtly affected the ROS level and percentage of apoptotic cells in blastocysts in this study, but these differences may be more substantial at different stages of development. Further research is necessary to clarify the mechanisms by which PD affects development of porcine oocytes.

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ABSTRACT IN KOREAN

본 연구는 체외성숙과정 동안 발생하는 산화스트레스에 대해서 Dioscorea 종에 주로 존재하는 스테로이드 사포닌인 프로토다이오신 (PD)의 처리가 돼지 난모세포의 손상을 완화시킬 수 있는지를 조사하였다. 적정농도를 선출하기 위하여 미성숙 난모세포의 성숙용 배양액에 여러가지 농도의 PD (0, 1, 10, 100, 200 μM)가 첨가되었으며, 좀 더 확실한 산화스트레스 모델을 위하여 200 μM 의 과산화수소도 추가로 첨가되었다. 발달능은 0 μM PD (control) 그룹에서 아무것도 처리하지 않은 normal group 에 비해 현저히 감소하였으며 10 μM 의 PD group(10PD)에서는 발달능의 감소가 나타나지 않았다. 활성산소 수치에는 세 그룹간에 큰 차이가 없었지만 글루타티온 수치와 항산화 유전자 (*SOD1*, *SOD2*, *Nrf2* 및 *HO-1*)의 mRNA 발현은 control 그룹에 비해 normal 그룹과 10PD 그룹에서 상당히 높게 나타났다. 또한, 10PD 그룹에서는 스핀들 형성들 형성 및 염색체 정렬에서의 결함이 적게 관찰되었으며, 전체 p44/42 에 대한 인산화된 p44/42 MAPK 비율이 10PD 그룹에서 높게 나타났다. 10PD 그룹에서 배반포 단계의 총 세포수는 control 그룹에 비해 크게 증가하였으며 세포사멸의 비율에는 큰 차이가 없었다. 발달과 관련된 유전자 (*POU5F1*, *CDX2* 및 *NANOG*)의 발현 역시 10PD 그룹에서 control 그룹보다 일관적으로 높게 나타났다. 이러한 결과는 PD 가 산화스트레스로부터 난모세포를 보호하며 발달능을 효과적으로 향상시킨다는 것을 나타낸다.

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