A Dissertation for the Degree of Master of Science

The antioxidant dieckol reduces damage of oxidative stress-exposed porcine oocytes and enhances subsequent parthenotes embryo development

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Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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항산화제 디에콜의 처리가 산화스트레스에 노출된 돼지 난자의 체외성숙 및 배아 발달에 미치는 영향

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편다빈

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ABSTRACT

This study investigated the effect of the antioxidant Dieckol, a component of ecklonia cava, on maturation and developmental competence of porcine oocytes exposed to oxidative stress *in vitro*. Oocytes were matured in *in vitro* maturation (IVM) medium containing various concentrations of dieckol. The blastocyst formation rate was highest in the 0.5 μM dieckol-treated (0.5 DEK) group. The reactive oxygen species level was decreased, and the level of glutathione and expression of antioxidant genes (*NFE2L, SOD1*, and *SOD2*) at metaphase II were increased in the 0.5 DEK group. Abnormal spindle organization and chromosome misalignment were prevented in the 0.5 DEK group. Expression of maternal markers (*CCNB1* and *MOS*) and activity of p44/42 mitogen-activated protein kinase was increased in the 0.5 DEK group. After parthenogenetic activation, the total number of cells per blastocyst was increased and the percentage of apoptotic cells was decreased in the 0.5 DEK group. Expression of development-related genes (*CX45, CDX2, POU5F1*, and *NANOG*), anti-apoptotic genes (*BCL2L1* and *BIRC5*), and a proapoptotic gene (*CASP3*) was altered in the 0.5 DEK group. These results indicate that the antioxidant dieckol improves IVM and subsequent development of porcine oocytes and can be used to improve the quality of oocytes under peroxidation experimental conditions.

Key words: *Dieckol, Porcine, IVM, Embryo, Oxidative stress*

1. INTRODUCTION

In vitro production (IVP) of embryos is an important biotechnology in reproductive therapy and biomedical research. IVP of oocytes is significantly less efficient than *in vivo* culture of oocytes. Consequently, culture conditions must be optimized to improve oocyte maturation and subsequent embryo development. *In vitro* maturation (IVM) is fundamental for IVP of embryos. Matured oocytes can be developed into embryos using spermatozoa or via parthenogenesis. During IVM, the cell cycle progresses to metaphase II (MII), and the nucleus and cytoplasm mature (Heikinheimo & Gibbons, 1998). Abnormal oocyte maturation perturbs embryo development. When overproduced, reactive oxygen species (ROS), such as hydroxyl ions, superoxide anions, and peroxyl radicals, cause oxidative damage to cellular components, disrupt regulation of signal transduction and gene expression, and alter activation of receptors and nuclear transcription factors (Chaube, Prasad, Thakur, & Shrivastav, 2005; Goud, Goud, Diamond, Gonik, & Abu-Soud, 2008). Antioxidative systems in oocytes and peripheral reproductive organs minimize oxidative damage by removing ROS *in vivo* (Guérin, El Mouatassim, & Ménézo, 2001). However, these systems are not established in mature oocytes *in vitro*, meaning excessive ROS can damage oocytes (Goto, Noda, Mori, & Nakano, 1993). To improve the production efficiency and quality of oocytes and embryos *in vitro*, a physiological level of intracellular ROS must be maintained. Many researchers are striving to define the intracellular environment and developmental competence of cells, oocytes, and embryos.

Ecklonia cava, a type of brown algae, is widely distributed around the southern coasts of Jeju Island in Korea and Japan. It contains a variety of compounds, including carotenoids, fucoidans, and phlorotannins, that have different biological activities (Kang et al., 2005). Among these components, phlorotannins, also called marine polyphenols, are a class of compounds with polymerized phloroglucinol units found in brown algae. Phlorotannins have several biological functions including anti-allergic, antibacterial, and antioxidant activities, and are anti-plasmin inhibitors (Nagayama et al., 2002; Sugiura et al., 2006). The antioxidant activities of single phlorotannins have been reported

(Kang et al., 2006; Kim et al., 2008), of which dieckol has the highest antioxidant activities. Dieckol displays high antioxidant activity in DPPH, hydroxyl, superoxide, and peroxyl radical scavenging experiments (Nakamura, Nagayama, Uchida, & Tanaka, 1996), inhibits proliferation and migration of lung cancer cells by regulating the phosphatidyl inositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway (Wang et al., 2019), attenuates type II diabetes in a mouse model (Kang et al., 2013), and protects against cytotoxicity by enhancing expression of antioxidant and detoxifying enzymes (Jeon et al., 2015).

Although dieckol has many biological activities, it is unknown whether it acts as an antioxidant in porcine oocytes. Accordingly, the present study investigated whether dieckol improves IVM of porcine oocytes and developmental competence of parthenotes embryos derived from these oocytes. We measured the level of oxidative stress and the efficiency of nuclear and cytoplasmic maturation in oocytes, and assessed blastocyst quality and expression of development-related genes. Based on the results, we propose that dieckol increases the production efficiency of porcine parthenotes embryos *in vitro* due to its antioxidative effect.

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. Oocyte collection and IVM

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 h 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, groups of 50 COCs were matured in 500 μL of 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 h at 38.8°C in an atmosphere of 5% CO_2 and 95% air. COCs were cultured in IVM medium containing 1% dimethyl sulfoxide and 0, 0.05, 0.5, 5, or 50 μM DEK for 44 h. In addition, 200 μM H_2O_2 was included to generate intracellular ROS.

2.3. PA and embryo culture

The denuded oocytes were used to evaluate oocyte survival and death. Survival of matured oocytes was estimated under a stereomicroscope based on morphological features such as oolemma

demarcation, intact zona pellucida, and cytoplasmic distribution. Following maturation, cumulus cells were removed by pipetting in the presence of 1 mg/mL hyaluronidase for 2–3 min. Oocytes were parthenogenetically activated with 5 μM Ca^{2+} ionomycin (Sigma) for 5 min. After 3 h of culture in porcine zygote medium (PZM)-5 containing 7.5 μg/mL cytochalasin B (Sigma), embryos were washed three times in PZM-5 containing 0.4% (w/v) BSA and cultured in the same medium for 6 days at 38.8°C in a humidified atmosphere of 5% CO_2 and 95% air. Oocytes and parthenotes embryos were washed in Dulbecco's phosphate-buffered saline (DPBS), and, depending on the experiment, either fixed in 4.0% (w/v) paraformaldehyde for 20 min and stored at 4°C or snap-frozen in liquid nitrogen and stored at -70°C.

2.4. Measurement of intracellular ROS and GSH levels

Dichlorohydrofluorescein diacetate (DCHFDA) and CellTracker™ Blue 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CMF2HC) 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 DCHFDA or 100 μM CellTracker™ Blue CMF2HC 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 determined using excitation and emission wavelengths of 450–490 nm and 515–565 nm, respectively. The excitation and emission wavelengths of CellTracker™ Blue CMF2HC are 371 and 464 nm, respectively. Grayscale images were acquired with a digital camera (Nikon, Tokyo, Japan) attached to the microscope. Mean grayscale values were calculated using Image J software. Background fluorescence values were subtracted from the final values before statistical analysis. The experiment was independently repeated six times with ten 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 h, and then oocytes were fixed overnight at 4°C with 4.0% (w/v) paraformaldehyde prepared in PBS. 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 calculated using ImageJ software (NIH, Bethesda, MD, USA). The experiment was independently repeated four times with 15–20 oocytes per experiment.

2.6. TUNEL assay and Hoechst staining

At 6 days after PA, blastocysts 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. 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 h at 38.8°C. Mitotic and apoptotic cells were scored. Nuclei were stained with Hoechst 33342 (1 μg/mL) for 30 min, and parthenotes embryos were washed with PBS containing 0.1% BSA. Blastocysts were mounted onto glass slides and examined under an inverted Olympus IX-71 fluorescence microscope. The experiment was independently repeated eight times with 5–12 blastocysts per experiment.

2.7. Western blot analysis

The protocol was basically the same as that described previously (S. E. Lee et al., 2012). In brief, oocytes (20–30 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 heated for 5 min at 95°C. Proteins were resolved on 5–12% Tris SDS-polyacrylamide electrophoresis gels for 1.5 h at 80–100 V. Samples were then transferred to HybondECL nitrocellulose membranes (Amersham, Buckinghamshire, UK) at 400 mA for 1.5 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) nonfat 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] 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 h with anti-rabbit IgG-horseradish peroxidase diluted 1:2,000 in blocking solution. After three washes with TBST, immunoreactive protein bands were visualized with a chemiluminescent reagent (Invitrogen). The experiment was independently repeated four times.

2.8. Real-time quantitative polymerase chain reaction

mRNA was isolated from more than three biological replicates, with 20–30 oocytes or 10–15 blastocysts per replicate, using a Dynabeads mRNA Direct Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. mRNA was collected in 10 μL elution buffers provided with the kit. Eluted RNA was reverse-transcribed into cDNA using an oligo (dT) 20 primer and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The protocol used was

basically the same as that described previously (S. E. Lee, Sun, 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 method (Livak & Schmittgen, 2001) after normalization against the expression level of a housekeeping gene (*ACTB*). The experiment was independently repeated 3–7 times.

Table 1. Primers used for quantitative polymerase chain reaction

Gene	GenBank accession no.	Primer sequences	Annealing temperature (°C)	Product size (bp)
ACTB	AY550069.1	F: AGATCATGTTCGAGACCTTC R: GTCAGGATCTTCATGAGGTAGT	54	220
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
BMP15	NM_001005155	F: CCCTCGGGTACTACACTATG R: GGCTGGGCAATCATATCCT	60	192
CCNB1	NM_001170768.1	F: CCAACTGGTTGGTGTCACTG R: GCTCTCCGAAGAAAATGCAG	60	195
GDF9	XQ68750.1	F: GTCTCCAACAAGAGAGAGATTC R: CTGCCAGAAGAGTCATGTTAC	54	109
MOS	NM_001113219	F: TGGGAAGAAACTGGAGGACA R: TTCGGGTCAGCCCAGTTCA	60	121
CX45	NM_001097519	F: CCCTCATAAGATAGACTGCT R: CTTCCAGTTCCCTCCTTTTA	54	170
CDX2	AM778830	F: AGCCAAGTGAAAACCAGGAC R: TGCGGTTCTGAAACCAGATT	60	178
POU5F1	NM_001113060	F: AGTGAGAGGCAACCTGGAGA R: TCGTTGCGAATAGTCACTGC	60	166
NANOG	DQ447201	F: TTCCTTCCTCCATGGATCTG R: ATCTGCTGGAGGCTGAGGTA	60	214

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
CASP3	NM_214131	F: GAGGCAGACTTCTTGTATGC R: CATGGACACAATACATGGAA	55	236

F, forward; R: reverse.

2.8. Statistical analysis

The general linear model procedure within the Statistical Analysis System (SAS User's Guide, 1985, Statistical Analysis System Inc., Cary, NC) was used to analyze data from all experiments. Tukey's multiple range test was used to determine significant differences. P-values less than 0.05 were considered significant. Values are presented as Mean ± SEM of independent experiments ($^{a\text{-}c}p < 0.05$, $^{*}p < 0.01$, and $^{**}p < 0.001$).

3. RESULTS

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

To determine the optimal concentration of dieckol, porcine oocytes were matured for 44 h in the presence of 0, 0.05, 0.5, 5, and 50 μM dieckol (control, 0.05 DEK, 0.5 DEK, 5 DEK, and 50 DEK, respectively) with or without (normal) 200 μM H_2O_2 to generate intracellular oxidative stress (Table 1). The percentage of surviving oocytes at the MII stage did not significantly differ among the normal, 0.05 DEK, and 0.5 DEK groups, but was significantly lower ($p < 0.05$) in the control and 50 DEK groups than in the other groups (normal, 84.0 ± 2.2%; control, 76.5 ± 3.5%; 0.05 DEK, 84.8 ± 3.2%; 0.5 DEK, 83.2 ± 1.4%; 5 DEK, 80.8 ± 3.6%; and 50 DEK, 52.7 ± 3.5%). Following parthenogenetic activation (PA), the percentage of cleaved oocytes was significantly higher ($p < 0.05$) in the normal group than in the other groups, did not significantly differ among the control, 0.05 DEK, 0.5 DEK, and 5 DEK groups, and was significantly lower ($p < 0.05$) in the 50 DEK group than in the control, 0.05 DEK, 0.5 DEK, and 5 DEK groups (normal, 73.1 ± 1.8%; control, 65.7 ± 1.5%; 0.05 DEK, 64.6 ± 0.9%; 0.5 DEK, 68.5 ± 0.7%; 5 DEK, 66.3 ± 2.2%; and 50 DEK, 47.6 ± 1.5%). The percentage of cleaved oocytes that reached the blastocyst stage on Day 6 was similar in the normal and 0.5 DEK groups, significantly lower ($p < 0.05$) in the control group than in the 0.5 DEK groups, significantly lower ($p < 0.05$) in the 50 DEK group than in the other groups, and did not significantly differ among the 0.05 DEK, 5 DEK and control groups (normal, 34.4 ± 2.1%; control, 21.3 ± 3.0%; 0.05 DEK, 26.8 ± 1.2%; 0.5 DEK, 35.2 ± 2.9%; 5 DEK, 31.3 ± 3.8%; and 50 DEK, 16.3 ± 0.5%). And the percentage of surviving oocytes that reached the blastocyst stage was also similar in the normal and 0.5 DEK groups, significantly lower ($p < 0.05$) in the control group than in the 0.5 DEK groups, significantly lower ($p < 0.05$) in the 50 DEK group than in the other groups, and did not significantly differ among the 0.05 DEK, 5 DEK and control groups (normal, 25.4 ± 1.2%; control, 16.2 ± 1.9%; 0.05 DEK, 17.2 ± 0.7%; 0.5 DEK, 24.0 ± 2.1%; 5 DEK, 20.4 ± 2.9%; and 50 DEK, 7.8 ± 0.3%). Therefore, 0.5 μ

M dieckol was selected as the optimal concentration, and the normal, control, and 0.5 DEK groups we re compared in subsequent experiments.

Table 2. Effects of different concentrations of dieckol on *in vitro* maturation of porcine oocytes

Group	Treatment (μM)		No. of GV stage oocytes	No. (%) of surviving oocytes	No. (%) of cleaved oocytes on Day 2	No. (%) of blastocysts per cleaved oocytes on Day 6	No. (%) of blastocysts per surviving oocytes
	H_2O_2	DEK					
Normal	0	0	150	127 (84.6 ± 2.0) ^{c}	93 (73.1 ± 1.8) ^{c}	32 (34.4 ± 2.1) ^{c}	25.4 ± 1.2 ^{c}
Control	200	0	150	114 (76.0 ± 3.5) ^{b}	75 (65.7 ± 1.5) ^{b}	16 (21.3 ± 3.0) ^{b}	16.2 ± 1.9 ^{b}
0.05 DEK	200	0.05	150	127 (84.8 ± 3.2) ^{c}	82 (64.6 ± 0.9) ^{b}	22 (26.8 ± 1.2) ^{b}	17.2 ± 0.7 ^{b}
0.5 DEK	200	0.5	150	124 (82.6 ± 0.8) ^{c}	85 (68.5 ± 0.7) ^{b}	30 (35.2 ± 2.9) ^{c}	24.0 ± 2.1 ^{c}
5 DEK	200	5	150	115 (80.6 ± 4.1) ^{bc}	76 (63.3 ± 3.7) ^{b}	24 (31.3 ± 3.8) ^{bc}	20.4 ± 2.9 ^{bc}
50 DEK	200	50	150	78 (52.0 ± 3.2) ^{a}	37 (47.6 ± 1.5) ^{a}	6 (16.3 ± 0.5) ^{a}	7.8 ± 0.3 ^{a}

Different superscript letters in the same column indicate values that significantly differ ($p < 0.05$).

3.2. Antioxidative effect of dieckol during IVM of porcine oocytes exposed to oxidative stress

We measured the levels of ROS, glutathione (GSH), and expression of antioxidant genes to assess the antioxidative effects of dieckol during IVM of porcine oocytes exposed to oxidative stress. The staining intensity of ROS was significantly lower ($p < 0.01$) in the normal and 0.5 DEK groups than in the control group (normal, 47.7 ± 1.8; control, 55.3 ± 1.8; and 0.5 DEK, 47.7 ± 2.1; Fig. 1b). The staining intensity of GSH was significantly higher ($p < 0.05$) in the 0.5 DEK group than in the control group, but did not significantly differ between the normal and other groups (normal, 88.1 ± 1.4; control, 83.0 ± 3.0; and 0.5 DEK, 92.2 ± 1.9; Fig. 1c). We next examined the effects of dieckol on expression of the antioxidant genes nuclear factor erythroid 2-like 2 (*NFE2L*), superoxide dismutase 1 (*SOD1*), and superoxide dismutase 2 (*SOD2*) (Fig. 1d). The mRNA levels of these genes were normalized against those in the normal group. mRNA expression of *NFE2L* and *SOD1* was significantly higher ($p < 0.05$) in the 0.5 DEK group than in the normal and control groups, *SOD2* differs between normal and 0.5 DEK group.

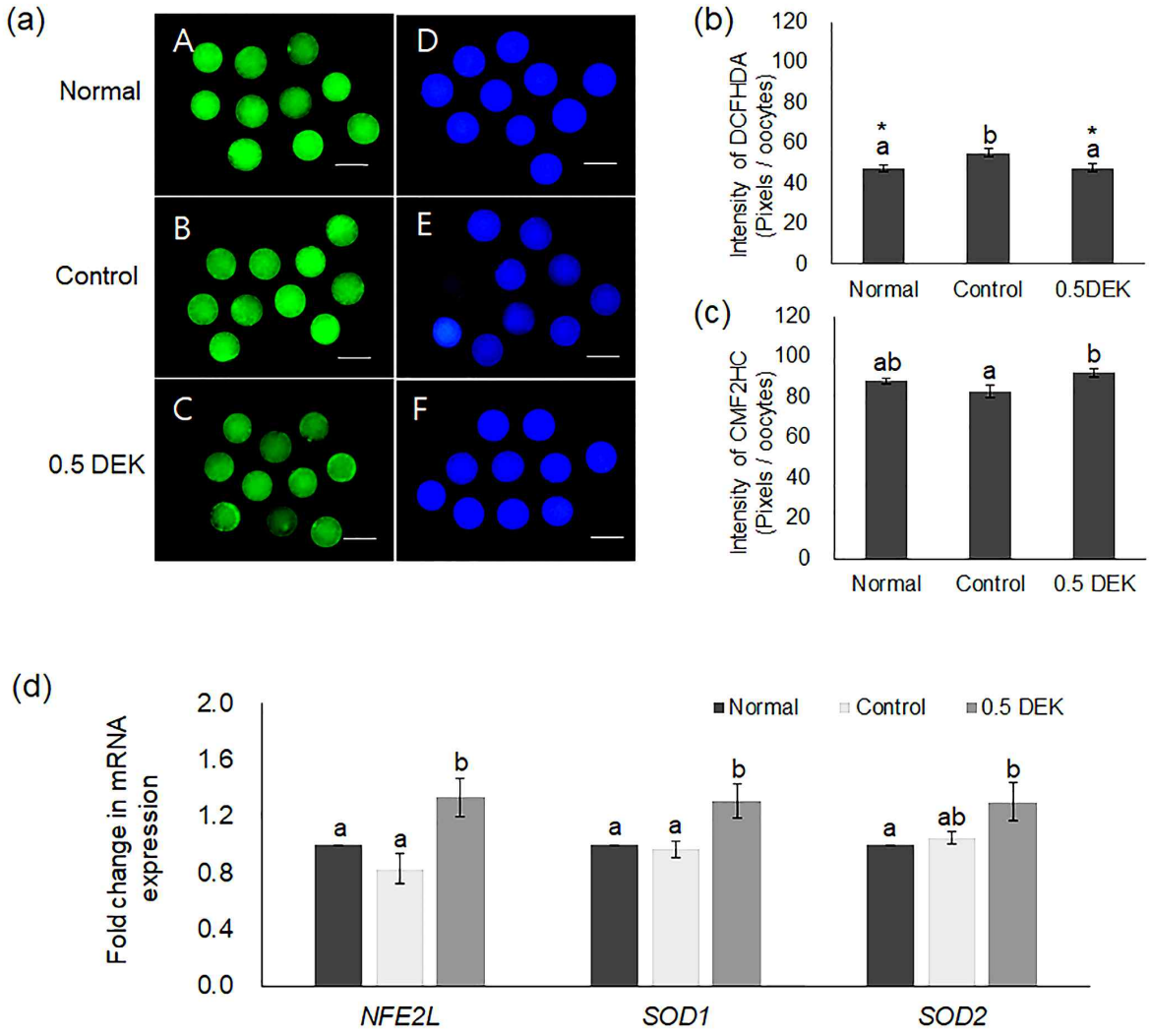

Fig. 1. Antioxidative effect of dieckol during porcine oocyte maturation. H_2O_2 was used to generate ROS. (a) Images of oocytes stained with DCHFDA (green) and CMF2HC (blue). (A–C) ROS staining; (D–F) GSH staining (scale bar, 100 μm). (b) Quantification of the fluorescence intensity of DCHFDA. (c) Quantification of the fluorescence intensity of CMF2HC. The experiment was independently repeated six times (n=60). (d) Relative expression of the antioxidant genes *NFE2L2, SOD1*, and *SOD2*. *ACTB* was used as an internal standard. Data were normalized against the corresponding levels in the normal group. The experiment was independently repeated seven times (n=175). Values are presented as Mean ± SEM of independent experiments ($^{a\text{-}b}p < 0.05$, $^{*}p < 0.01$).

3.3. Dieckol increases the levels of molecular maturation factors in porcine oocytes exposed to oxidative stress *in vitro*

To examine the effect of dieckol on cytoplasmic maturation of oocytes, we measured p44/42 MAPK activity and maternal gene expression. Western blot analysis revealed that phospho-p44/42 MAPK, the active form of this kinase, migrated as a doublet in lysates of matured porcine oocytes (Fig. 3a). The ratio of phospho-p44/42 MAPK to p44/42 MAPK was normalized against that in the normal group. This ratio was significantly higher ($p < 0.01$) in the 0.5 DEK group than in the normal and control groups (normal, 1.00 ± 0.00; control, 0.98 ± 0.03; and 0.5 DEK, 1.23 ± 0.05). Expression of the maternal genes growth differentiation factor-9 (*GDF9*), cyclin B1 (*CCNB1*), bone morphogenetic protein 15 (*BMP15*), and MOS proto-oncogene, serine/threonine kinase (*MOS*) at the MII stage was analyzed by real-time RT-PCR (Fig. 3b). Expression of *BMP15* was increased in the 0.5 DEK group, but did not significantly differ among the groups. Expression of *CCNB1* and *MOS* was significantly higher ($p < 0.01$ and $p < 0.05$, respectively) in the 0.5 DEK group than in the normal and control groups. Expression of *GDF9* was significantly higher ($p < 0.05$) in the 0.5 DEK group than in the normal group, but did not differ between the control group and the 0.5 DEK and normal groups.

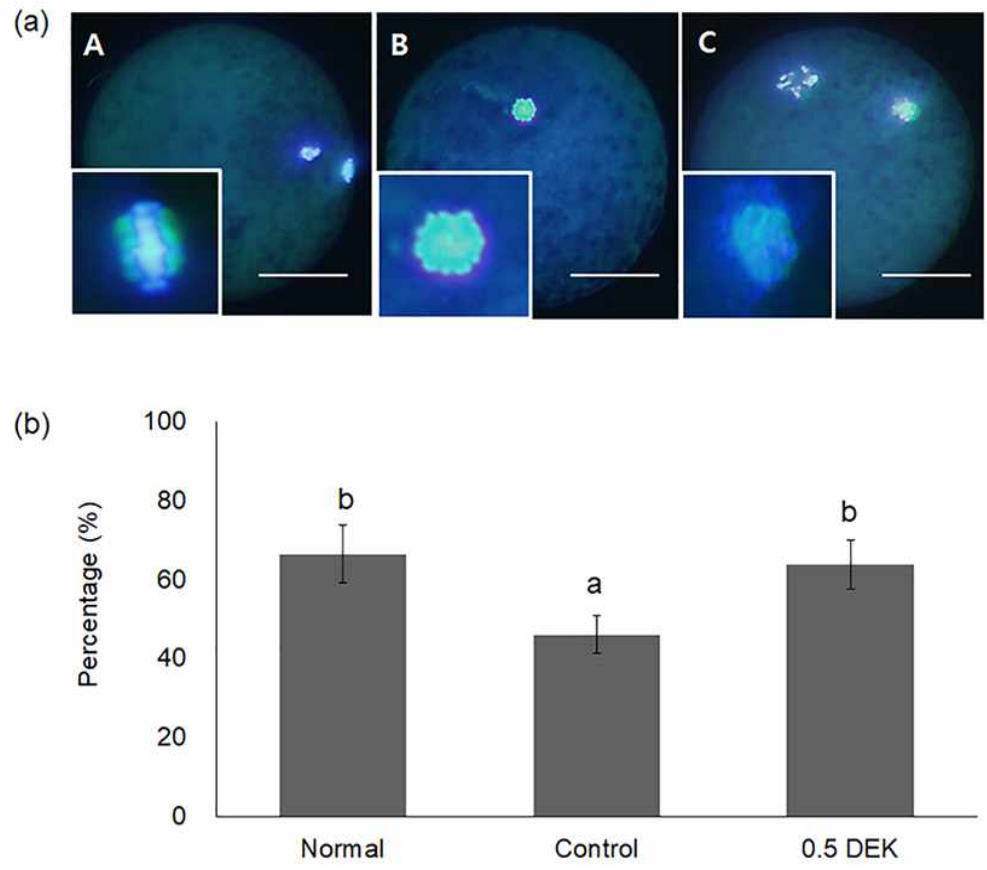

Fig. 2. Effect of dieckol on chromosome alignment and spindle morphology in porcine oocytes *in vitro*. (a) Normal (A) and abnormal (B–C) chromosome alignment and meiotic spindle formation in oocytes (scale bar, 50 μm). (b) Percentage of oocytes with normal chromosome alignment and meiotic spindle morphology. The experiment was independently repeated four times (normal group, n=87; control group, n=91; 0.5 DEK group, n=97; $^{a\text{-}b}p < 0.05$).

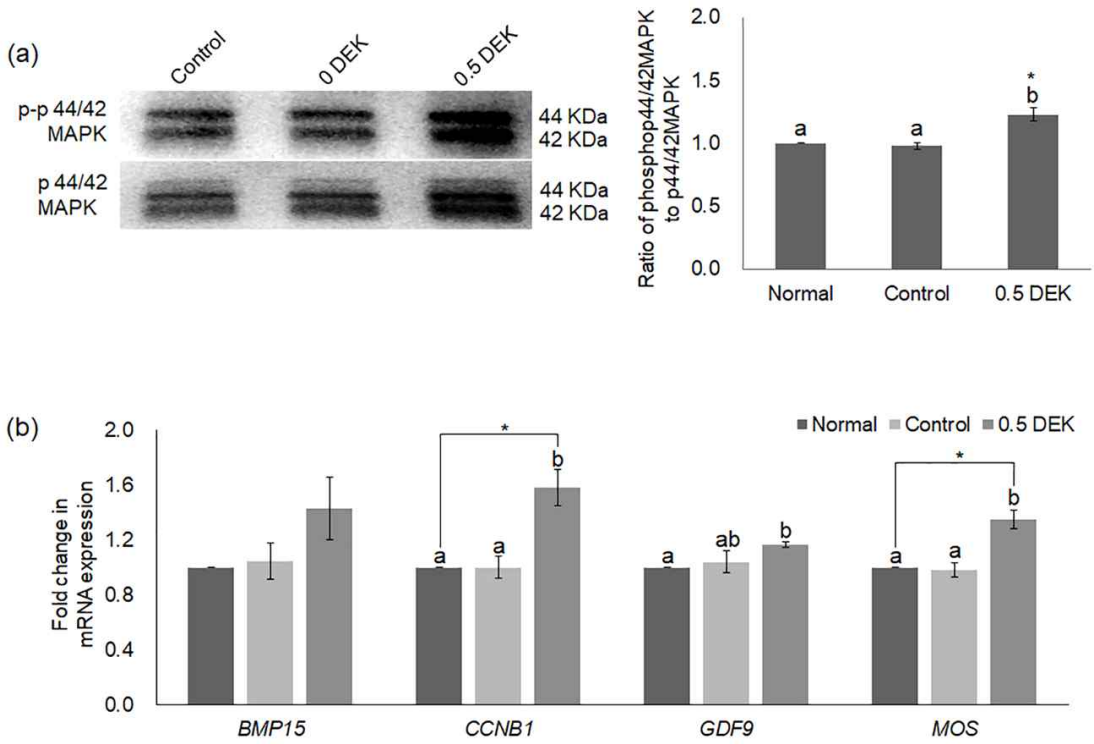

Fig. 3. Effects of dieckol on cytoplasmic maturation of porcine oocytes *in vitro*. (a) Western blotting of total and phosphorylated p44/42 MAPK, and the ratio of phospho-p44/42 MAPK to p44/42 MAPK. The experiment was independently repeated four times (n=115). (b) Maternal gene expression. *ACTB* was used as an internal standard. Data were normalized against the corresponding levels in the normal group. The experiment was independently repeated three times (n=90). Values are presented as Mean ± SEM of independent experiments ($^{a-b}p < 0.05$, $*p < 0.01$).

3.4. Dieckol improves the developmental ability and quality of parthenotes embryos derived from porcine oocytes exposed to oxidative stress *in vitro*

To investigate the effect of dieckol during IVM of porcine oocytes, we assessed the developmental capacity and quality of parthenotes embryos derived from these oocytes. To assess embryo development, blastocysts were classified into the early, middle, expanding, and hatching/hatched stages, and the percentages of blastocysts at each stage were determined (Fig. 4a and b). We classified blastocyst stage as early-hatching blastocyst as described previously (Thompson, Onwubalili, Brown, Jindal, & McGovern, 2013). The percentage of all blastocysts was significantly higher ($p < 0.05$) in the normal and 0.5 DEK groups than in the control group (normal, 51.0 ± 3.2%; control, 40.3 ± 4.1%; and 0.5 DEK, 54.6 ± 2.4%). The percentage of early-stage blastocysts was decreased in the normal and 0.5 DEK groups. The percentage of middle-stage blastocysts did not differ among the groups. The percentage of expanding-stage blastocysts was higher in the normal and 0.5 DEK groups than in the control group. The percentage of hatching/hatched-stage blastocysts did not significantly differ among the groups. The total number of cells per blastocyst was significantly higher ($p < 0.05$) in the 0.5 DEK group than in the control group, and did not differ between the normal group and 0.5 DEK groups (normal, 63.7 ± 2.0; control, 59.5 ± 1.8; and 0.5 DEK, 65.3 ± 1.8; Fig. 4c). Genomic DNA fragmentation was assessed by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) to detect apoptotic cells. The percentage of apoptotic cells was significantly lower ($p < 0.001$) in the normal and 0.5 DEK groups than in the control group (normal, 2.0 ± 0.3%; control, 5.7 ± 0.7%; and 0.5 DEK, 2.7 ± 0.7%; Fig. 4d).

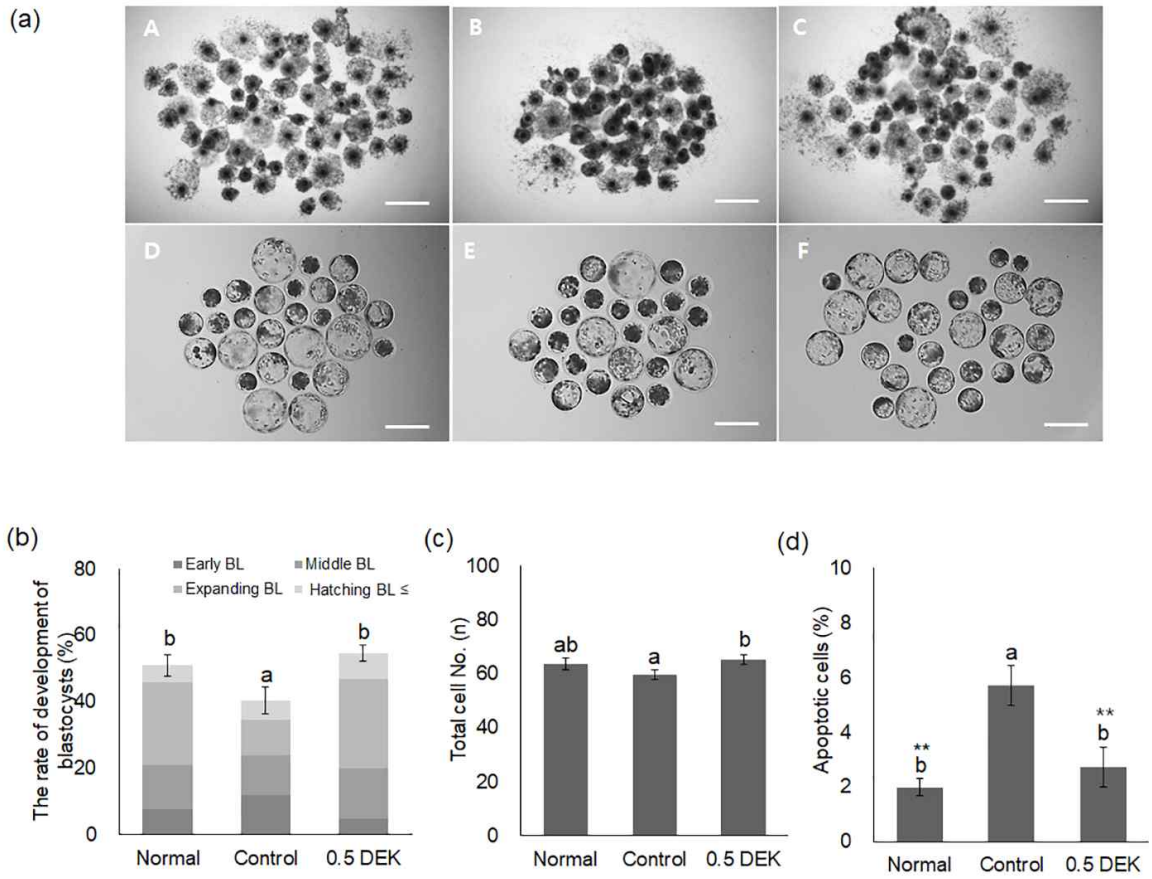

Fig. 4. Effect of dieckol treatment during IVM of porcine oocytes on subsequent embryo development. Blastocysts were fixed on Day 6. (a) The degree of cumulus cell expansion (A–C) and morphology of blastocysts (D–F) (A, D, normal group; B, E, control group; and C, F, 0.5 DEK group) (scale bar, 200 μm). (b) Percentages of blastocysts at the early, middle, expanding, and hatching/hatched stages (BL, Blastocyst). Error bars represent the Mean ± SEM. (c) Total number of cells per blastocyst. (d) Percentage of apoptotic cells in blastocysts. The experiment was independently repeated eight times (normal group, n=59; control group, n=56; 0.5 DEK group, n=68; $^{a-b}p < 0.05$, $*p < 0.01$, and $**p < 0.001$).

3.5. Dieckol treatment of porcine oocytes exposed to oxidative stress *in vitro* affects relative expression of developmentally important genes at the blastocyst stage

To investigate the effect of dieckol during IVM of porcine oocytes, we assessed the developmental capacity and quality of parthenotes embryos derived from these oocytes. To assess embryo development, blastocysts were classified into the early, middle, expanding, and hatching/hatched stages, and the percentages of blastocysts at each stage were determined (Fig. 4a and b). We classified blastocyst stage as early-hatching blastocyst as described previously (Thompson, Onwubalili, Brown, Jindal, & McGovern, 2013). The percentage of all blastocysts was significantly higher ($p < 0.05$) in the normal and 0.5 DEK groups than in the control group (normal, 51.0 ± 3.2%; control, 40.3 ± 4.1%; and 0.5 DEK, 54.6 ± 2.4%). The percentage of early-stage blastocysts was decreased in the normal and 0.5 DEK groups. The percentage of middle-stage blastocysts did not differ among the groups. The percentage of expanding-stage blastocysts was higher in the normal and 0.5 DEK groups than in the control group. The percentage of hatching/hatched-stage blastocysts did not significantly differ among the groups. The total number of cells per blastocyst was significantly higher ($p < 0.05$) in the 0.5 DEK group than in the control group, and did not differ between the normal group and 0.5 DEK groups (normal, 63.7 ± 2.0; control, 59.5 ± 1.8; and 0.5 DEK, 65.3 ± 1.8; Fig. 4c). Genomic DNA fragmentation was assessed by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) to detect apoptotic cells. The percentage of apoptotic cells was significantly lower ($p < 0.001$) in the normal and 0.5 DEK groups than in the control group (normal, 2.0 ± 0.3%; control, 5.7 ± 0.7%; and 0.5 DEK, 2.7 ± 0.7%; Fig. 4d).

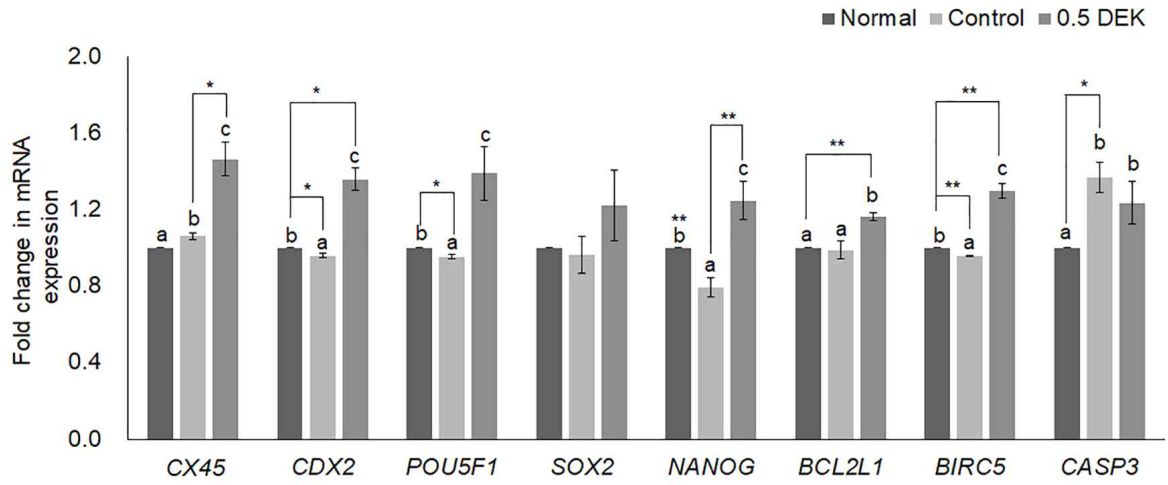

Fig. 5. Effect of dieckol on relative expression of development- and apoptosis-related genes. Relative mRNA expression of development-related genes (*CX45, CDX2, POU5F1, SOX2*, and *NANOG*), anti-apoptotic genes (*BCL2L1* and *BIRC5*), and a pro-apoptotic gene (*CASP3*) at the blastocyst stage. *ACTB* was used as an internal standard. Data were normalized against the corresponding levels in the normal group. The experiment was independently repeated three times. Values are presented as Mean ± SEM of independent experiments ($^{a\text{-}c}p < 0.05$, $*p < 0.01$, and $**p < 0.001$).

4. DISSCUSION

We investigated the effect of dieckol on IVM of porcine oocytes exposed to oxidative stress and the developmental capacity of parthenotes embryos derived from these oocytes. Of course, the parthenogenetic experiment method is limited and different from general embryonic development because it generates embryos without sperm may have different mechanisms. But nowadays, in vitro studies such as genetically engineered embryo cloning experiments as well as artificial fertilization are being actively conducted, and a parthenogenetic experiment can be used as an experimental model for establishing such an experimental system. Accordingly, result show that dieckol treatment increased the GSH level and antioxidant gene expression, prevented chromosome misalignment, and increased MAPK activity and expression of maternal genes in oocytes. Moreover, dieckol treatment decreased the percentage of apoptotic cells, increased the total cell number, and altered expression of developmentally important genes in blastocysts.

Porcine oocytes were matured *in vitro* for 44 h in the presence of various concentrations of dieckol (0, 0.05, 0.5, 5, or 50 μM) and 200 μM H_2O_2. The survival rate of oocytes was higher in the 0.5 DEK group than in the control group, and the blastocyst formation rate was highest in the 0.5 DEK group (Table 1). The efficiency of IVM must be improved because oocyte maturation is important for control of cellular metabolism during meiosis and is the basis of blastocyst growth prior to embryonic development. Several studies have shown that supplementation of various antioxidants promotes oocyte maturation and improves development *in vitro* (Hennings et al., 2016; Y. Jeon et al., 2014). Tannic acid exhibits antioxidant activity by scavenging the DPPH free radical and the ABTS radical (Gülçin, Huyut, Elmastaş, & Aboul-Enein, 2010), and inhibits iron-dependent free radical formation *in vitro* (Andrade et al., 2006). During IVM of porcine oocytes, melatonin supplementation increases the polar extrusion rate and elicits a beneficial effect (Kang et al., 2009). Supplementation of melatonin and tannic acid together increases the cleavage and blastocyst formation rates (Lane &

Whitaker, 2018). We investigated the optimal concentration for processing in porcine oocytes of dieckol. In raw data, we found that all oocytes died after IVM when treated with 500 uM dieckol. The results confirmed that too high an antioxidant concentration negatively affects oocytes. It is important to treat the antioxidants to protect the oocytes during IVM, but it is also important to maintain the proper balance of antioxidants according to the concentration setting. Therefore, our results demonstrated that low concentrations of Dieckol were effective in protecting the damage caused by oxidative stress in oocytes. Therefore, we used dieckol at a concentration of 0.5 μM and demonstrated that its antioxidative effect improved the blastocyst formation rate by promoting oocyte maturation with MII.

The intracellular oxidative and antioxidative statuses are primarily determined by ROS and GSH activity. Many studies have reported changes in the oxidant-antioxidant balance following addition of H_2O_2 to IVM medium (Tu et al., 2016; Nguyen et al., 2017; Truong & Gardner, 2017; Yao et al., 2019). External oxygen or an inefficient antioxidant system increases ROS generation *in vitro* (Armand et al., 2019). ROS are generated as a natural byproduct of cellular metabolism and play important roles in cell signaling and homeostasis (Devasagayam et al., 2004). However, an excessive increase in the ROS level interferes with mitochondrial function directly or indirectly (W. Zhang & Liu, 2002). GSH is a cofactor of glutathione peroxidase and glutathione-S-transferase and plays an important role in protection of cellular lipids, proteins, and nucleic acids against oxidative stress caused by ROS (Gérard-Monnier & Chaudiere, 1996). An imbalance in the intracellular antioxidant regulatory system can damage various molecules in oocytes and decrease blastocyst quality (Miao et al., 2019). In the current study, dieckol elicited antioxidative effects in oocytes exposed to oxidative stress by reducing the ROS level and increasing the GSH level (Fig. 1b and c). In addition, dieckol increased antioxidant gene expression in comparison to normal group and oxidant stress group (Fig. 1d). *NFE2L* is a transcription factor that controls expression of antioxidants and detoxifying enzymes. The *NFE2L* signaling system is the most important cellular defense and survival pathway against oxidative stress and toxicants (Ryoo & Kwak, 2018; H. Zhang, Davies, & Forman, 2015). *NFE2L*

activated by this pathway promotes expression of the antioxidants *SOD1* and *SOD2* (Dong, Sulik, & Chen, 2008). Dieckol is an oligomer of phloroglucinol attached via C-C or C-O-C bonds, (Lüder & Clayton, 2004) and elicits cytoprotective, photoprotective, and antioxidative effects (Lee, Kim, Yoo, & Kwon, 2013; Pallela, Na-Young, & Kim, 2010). Dieckol exerts cytoprotective effects by enhancing expression of antioxidants and phase II detoxifying enzymes via activation of the *NFE2L*-ARE pathway through phosphorylation of MAPKs, especially ERK (M. S. Lee et al., 2015). The signal transduction proteins MAPK and PI3K/Akt facilitate nuclear translocation and transcriptional activation of *NFE2L* (Nguyen, Nioi, & Pickett, 2009). Dieckol counterbalances oxidative stress during IVM of porcine oocytes and protects cells against oxidative stress by inducing production of antioxidants, which reduce the levels of cytotoxic substances and scavenge free radicals.

The meiotic spindle forms in the oocyte at the MII stage after segregation of bivalent chromosomes and extrusion of the first polar body. Generation of excessive ROS at this time alters the levels of spindle-related proteins (Cecconi et al., 2014) and induces acetylation of α-tubulin (Lee et al., 2013). Aristolochic acid is a type of carcinogenic and nephrotoxic nitrotenan carboxylic acid. Exposure to this substance causes DNA damage and marked spindle defects in oocytes due to excessive oxidative stress and interferes with meiotic progression (Y. Zhang et al., 2019). Normal chromosomal alignment and meiotic spindle formation can be used to assess nuclear maturation of oocytes. Dieckol treatment increased the percentage of oocytes with these normal features (Fig. 2) and MAPK activity and maternal gene expression (Fig. 3a and b) were increased in the 0.5 DEK group in comparison to normal group, or control. MAPK regulates cell cycle progression by modulating microtubules and actin filaments, is activated at the germinal vesicle breakdown stage (GVBD) of oocyte maturation, and is essential for resumption of meiosis and maintenance of meiotic arrest (Villa-Diaz & Miyano, 2004). MAPK activity is high in porcine oocytes cultured in IVM medium supplemented with the cytokines FGF2, LIF, and IGF1, which improve oocyte maturation (Yuan et al., 2017). MAPK activity improves bovine oocyte maturation *in vitro* (Gordo, He, Smith, & Fissore, 2001). *CCNB1* and *MOS* are involved in regulation of MAPK and maturation-promoting factor, which are important meiotic regulators (Liang, Su, Fan, Schatten, & Sun, 2007; Sánchez &

Smitz, 2012). Expression of these two genes was significantly higher in the dieckol treatment than normal group and control. Taken together, these results show that dieckol elicits antioxidative effects during IVM of oocytes exposed to oxidative stress and improves oocyte maturation by increasing the levels of nuclear maturation and molecular maturation factors and promoting progression to the MII stage.

We further investigated whether dieckol affects the quality of parthenotes embryos derived from oocytes following PA and culture *in vitro* for 6 days. Blastocysts were divided into the early, middle, expanding, and hatching/hatched stages. The percentage of blastocysts at the expanding stage was increased in the 0.5 DEK group, while the percentage of blastocysts at the early stage was decreased in comparison to the control. Furthermore, the total cell number per blastocyst was increased and the percentage of apoptotic cells was decreased in the 0.5 DEK group than control (Fig. 4c and d). Acceleration of embryo development is indicative of improved embryo quality, and the total cell number is an indicator of blastocyst quality (Luna et al., 2008). An increase in trophectoderm (TE) cells facilitates formation of a healthy placenta, while the inner cell mass (ICM) is essential for a successful pregnancy and live birth (Zhao, Yu, & Zhang, 2018). Serum supplementation yields a high blastocyst development rate and cell number per blastocyst, and promotes embryonic cell division and development by facilitating progression from the early morula stage to the blastocyst stage (Holm, Booth, & Callesen, 2002). Excessive oxidative stress induces apoptosis and reduces the oocyte developmental capacity (Goud et al., 2008). The antioxidant PGE2, which stimulates antioxidant gene expression and reduces the intracellular levels of free radical species, improves the mitochondrial membrane potential and reduces apoptosis (Boruszewska et al., 2020). We examined relative expression of developmentally important genes (*CX45, CDX2, POU5F1, SOX2, NANOG, BCL2L1, BIRC5,* and *CASP3*). Tight junctions play a major role in oocyte maturation and meiotic resumption by directing the transfer of numerous molecules between oocytes. Zygotes and individual blastomeres are totipotent, meaning that a single cell is likely to develop into an embryo. The placenta must support the cells that constitute the fetus. Differentiation of pluripotent cells and separation into distinct TE and ICM cell lineages begin at the blastocyst stage. *CDX2* is an early transcription factor

essential for formation and maintenance of the TE cell lineage in mammalian embryos (Niwa et al., 2005; Yamanaka, Ralston, Stephenson, & Rossant, 2006). *CX45* regulates gap junction communication. Little is known about the oocyte factors that direct formation of the ICM and TE. The transcription factors *POU5F1, SOX2*, and *NANOG* regulate transcription in embryonic stem cells (Avilion et al., 2003; Chambers et al., 2003). *In vitro* culture of blastocysts at an oxygen tension of 20%, which is similar to the atmospheric oxygen concentration, alters the profile of these three transcription factors, which are associated with the expression levels of early development genes, and causes DNA fragmentation (Leite et al., 2017). Dieckol upregulated mRNA expression of development-related genes (*CX45, CDX2, POU5F1* and *NANOG*) and altered expression of apoptosis-related genes (Fig. 5). Members of the BCL-2 family are apoptosis regulators that function in a wide variety of cellular activities. *BIRC5* inhibits caspase activation and thereby negatively regulates programmed cell death. Treatment with stem cell factor, a growth factor that reduces cellular apoptosis, increases *BCL2L1* expression, while anti-apoptotic genes are poor expressed in degenerative embryos (Felici et al., 1999). A recent study reported that treatment of *in vitro* fertilized porcine oocytes with mesenchymal stem cell-derived bioactive material (hAT-MSC-BM) and human embryonic stem cell-derived bioactive material (hESC-BM) increases expression of transcription factors including *POU5F1* and *SOX2*, as well as anti-apoptotic genes including *BCL2L1* and *BIRC5* (S. E. Lee, Moon, Kim, & Park, 2015). Another study reported that *TNFAIP2, BAX, POU5F1* and *BCL2L1* are highly expressed in zinc-treated *in vitro* fertilized blastocysts (Y. Jeon et al., 2014). Abundant gene expression is one of the main checkpoints for high-quality embryo development and its disturbance can limit blastocyst production. In summary, our experiments demonstrate that the antioxidative effect of dieckol not only increases the blastocyst formation rate and the total number of cells per blastocyst, but also inhibits apoptosis in parthenotes embryos derived from oxidative stress-induced porcine oocytes and regulates expression of transcription factors important for parthenotes embryo development.

In conclusion, excessive ROS disrupt intracellular molecular function during IVM of porcine oocytes and thereby limit maturity, resulting in production of low-quality blastocysts. Dieckol is a

marine phlorotannin and potent antioxidant. Our results demonstrate that supplementation of IVM medium with dieckol protects oocytes against oxidative stress, promotes nuclear and cytoplasmic maturation, and improves blastocyst formation and development, and thereby facilitates the development of good-quality parthenotes embryos. Thus, the antioxidant dieckol can be used to establish more efficient experimental models by preventing oxidative stress of oocytes cultured *in vitro* during the production of assisted reproductive technology (ART) and animal cloning experiments.

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ABSTRACT KOREAN

이 연구는 감태의 주성분인 항산화제 디에콜이 체외에서 산화스트레스에 노출된 돼지 난자의 성숙과 발달능력에 미치는 영향을 조사했다. 돼지 난소로부터 회수된 미성숙 난자는 다양한 농도의 디에콜을 함유하는 체외성숙용 배양액에서 배양되었다. 그 결과 0.5 μM의 디에콜을 처리 (0.5 DEK) 그룹에서 배반포 형성률이 가장 높았다. 또한, 0.5 DEK 그룹에서 활성산소종의 수준이 감소하고 글루타티온 수준과 항산화 유전자 (*NFE2L, SOD1, SOD2*)의 발현이 증가하였다. 0.5 DEK 그룹에서 비정상적인 방추사의 형성과 염색체 오정렬이 방지되었다. 모체유전자 (*CCNB1, MOS*)의 발현과 p44/42 MAPK의 활성이 증가 됨을 확인할 수 있었다. 단위발생을 유도한 후, 배반포 당 총 세포 수가 0.5 DEK 그룹에서 증가하였으며, 사멸 세포의 비율은 감소하였다. 0.5 DEK 그룹에서 배아 발달과 관련한 유전자 (*CX45, CDX2, POU5F1* 및 *NANOG*)의 발현이 증가하였으며, 항 세포사멸 유전자 (*BCL2L1, BIRC5*) 및 프로-세포사멸 유전자 (*CASP3*)의 발현이 조절되었다. 이러한 결과는 산화 방지제 디에콜이 돼지 난자의 체외성숙 및 후속 배아 발달을 개선하고 산화스트레스에 노출되기 쉬운 실험 환경에서 난자의 품질 향상에 이용될 수 있음을 증명한다.

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