A Dissertation for the Degree of Master of Science

Isorhamnetin improves *in vitro* maturation of oxidative stress-exposed porcine oocytes and subsequent embryo development

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Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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A thesis submitted as a Qualified Dissertation

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ABSTRACT

The flavonoid-based compound isorhamnetin (ISO) is a 3'-methoxylated derivative of quercetin. This study investigated the effects of various concentrations of ISO on in vitro maturaion (IVM) of porcine oocytes exposed to oxidative stress (200 μM hydrogen peroxide). We examined oxidative stress level, maturation efficiency, cumulus cell expansion, and expression of maturation-related factors during oocyte maturation and subsequent embryo developmental competence and blastocyst quality. Treatment with 2 μM ISO (2 ISO) increased the percentages of surviving oocytes, and cleaved embryos on day 2 and blastocysts on day 7. The glutathione level and mRNA expression of antioxidant-related genes (NFE2L2 and SOD2) were increased in the 2 ISO-treated group, while the reactive oxygen species level was decreased. Treatment with 2 ISO increased mRNA expression of a cumulus cell expansion-related gene (SHAS2) and improved chromosomal alignment. mRNA expression of maternal genes (CCNB1, MOS, BMP15, and GDF9) and MAPK activity were increased in the 2 ISO-treated group. The total cell number per blastocyst and percentage of apoptotic cells were increased and decreased in the 2 ISO-treated group, respectively. Treatment with 2 ISO increased mRNA expression of development-related genes (SOX2, NANOG, and POU5F1) and anti-apoptotic genes (BCL2L1, BIRC5, and BRIC3), and decreased that of pro-apoptotic genes (CASP3 and FAS). These results demonstrate that 2 ISO improves the quality of porcine oocytes by protecting them against oxidative stress during IVM and enhances subsequent embryo development in vitro. Therefore, we propose that ISO is a useful supplement for IVM of porcine oocytes.

Key words: Porcine, Oocyte, Oxidative Stress, Isorhamnetin, Antioxidant

1. INTRODUCTION

In vitro maturation (IVM) is a technique that allows oocytes to mature in vitro and can be performed for women with fertility problems. Matured oocytes arrest at metaphase of the second meiotic division (MII) and are activated by spermatozoa or an artificial stimulus. After activation, meiotic division resumes and embryo development begins. However, in vitro-matured oocytes have a variable lack compared with in vivo matured oocytes. Multiple factors contribute to the poor quality of in vitro-matured oocytes. One important factor is oxidative stress (Khazaei & Aghaz, 2017) because the oxygen concentration is higher in vitro than in vivo (Tatemoto, Sakurai, & Muto, 2000). Consequently, attempts have been made to improve IVM efficiency by protecting oocytes against oxidative stress.

Oxidative stress naturally arises because superoxide (O^2-) and hydrogen peroxide (H_2O_2) form during metabolic processes (Storz & Imlay, 1999). These chemically reactive species containing oxygen are called reactive oxygen species (ROS). An increase in intracellular ROS leads to lipid peroxidation (Mihalas, De Iuliis, Redgrove, McLaughlin, & Nixon, 2017), DNA damage (Menezo, Dale, & Cohen, 2010), and inhibition of meiotic maturation (Ambruosi et al., 2011) in oocytes. The ROS level in porcine oocytes increases upon heat stress (Itami, Shirasuna, Kuwayama, & Iwata, 2018), aging (W. J. Kim et al., 2019), endoplasmic reticulum stress (Park et al., 2018), and hyperoxia (Goud, Goud, Diamond, Gonik, & Abu-Soud, 2008) conditions. Many researchers have supplemented IVM medium with antioxidants to reduce damage under these conditions.

The antioxidant isorhamnetin (ISO) is a 3'-methoxylated derivative of quercetin and a flavonoid-based compound. It has anticancer (J. E. Kim et al., 2011), anti-inflammatory (Boesch-Saadatmandi et al., 2011), and antioxidative (Pengfei, Tiansheng, Xianglin, & Jianguo, 2009) activities and inhibits HO-induced activation of the apoptotic pathway (B. Sun et al., 2012). ISO protects cells from ROS by inducing expression of nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent antioxidant genes (Yang et al., 2014). Quercetin, which has a similar structure to ISO, has been widely studied in the medical and biological fields, but ISO has not been well investigated. We hypothesized that

supplementation of ISO may elicit beneficial effects during IVM of porcine oocytes due to its antioxidant activity.

The present study investigated the effects of treatment with various concentrations of ISO during IVM of porcine oocytes exposed to oxidative stress via H2O2 treatment. We examined the developmental rate, ROS level, cumulus cell expansion, and maturation factor expression during IVM, as well as subsequent embryo developmental competence and blastocyst quality. We speculate that ISO improves the maturation, developmental competence, and quality of embryos derived from oxidative stress-exposed oocytes in vitro and can be used to improve the efficiency of porcine embryo production.

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 32–35°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). Group of 50 COCs were matured 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 h at 38.8°C in a humidified atmosphere of 5% CO_2 in air.

COCs were cultured in IVM medium containing 1% dimethyl sulfoxide, 0, 0.02, 0.2, 2, or 20 ISO, and 200 μM H_2O_2 for 44 h. The experiment was independently repeated seven times, with 50 oocytes per experiment.

2.3. PA and embryo culture

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 7 days at 38.8°C in a humidified atmosphere of 5% CO_2 in air.

2.4. Measurement of intracellular ROS and GSH levels

DCFH-DA and CellTracker™ Blue CMF_2HC were used to determine the intracellular levels of ROS and GSH, respectively, as previously described (Yang et al. 1998; You et al. 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 Dulbecco's phosphate-buffered saline (DPBS) containing 50 μM DCFH-DA 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 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 CMF_2HC 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 ImageJ software (NIH, Bethesda, MD, USA). Background fluorescence values were subtracted from the final values prior to statistical analysis. The experiment was independently repeated five 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 h, and then oocytes were fixed overnight at 4°C with 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 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. At least 20 oocytes were examined per group.

2.6. Hoechst staining

Blastocysts were cultured for 7 days after PA, fixed overnight at 4°C in 4.0% (w/v) paraformaldehyde prepared in PBS, washed more than three times with PBS containing 0.1% BSA, and incubated with 1 μg/mL Hoechst 33342 at 38.8°C for 30 min. Thereafter, blastocysts were washed with PBS containing 0.1% BSA, mounted onto glass slides, and examined under an epifluorescence microscope. The experiment was independently repeated seven times, and at least 10 blastocysts were examined per group.

2.7. Western blot analysis

The protocol was basically the same as that described previously (S. E. Lee, Sun, Choi, Uhm, & Kim, 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 gel 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.

2.7. Real-time quantitative polymerase chain reaction

Real-time RT-PCR was performed as described previously (Lee et al. 2014). mRNA was isolated from groups of 20 *in vitro*-matured oocytes using a Dynabeads mRNA Direct Kit (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 2 μg mRNA per sample using an oligo $(dT)_{20}$ primer and SuperScript III reverse transcriptase (Invitrogen). 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 40 cycles of 15 sec at 95°C and 60 sec at 55°C or 60°C. Samples were then cooled to 12°C. Relative gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) after normalization against the mRNA level of *ACTB*. The experiment was independently repeated 4–6 times.

Table 1. Primers used for quantitative polymerase chain reaction

Gene	Gene Bank accession number	Primer sequence	Annealing temperature (°C)	Product size (bp)
ACTB	AY550069.1	F : AGATCATGTTCGAGACCTTC R : GTCAGGATCTTCATGGGTAGT	54	220
NFE2L2	Gu991000.1	F : TGGAGTGTACACGTTTCTGT R : GTGTCTGTGATCTTGTCCAG	54	99
SOD1	GU944822.1	F : GTGTTAGTAACGGGAACCAT R : GGATTCAGGATTGAAGTGAG	54	173
SOD2	NM_214127.2	F : AGACCTGATTACCTGAAAGC R : CTTGATGTACTCGGTGTGAG	54	110
CTSB	NM_001097458.1	F : CTCTAGGAACGAGAAGGAGAT R : CCAGACTTATACTGCAGGAAG	54	99
SHAS2	NM_213053.1	F : ACTGTCCAGTTAGTAGGTCTCA R : ACATGTACAACACCGAGTAGAG	54	100
SOX2	EU503117	F : GCCCTGCAGTACAACTCCAT R : GCTGATCATGTCCCGTAGGT	60	216
NANOG	DQ447201.1	F : GAACTTTCCAACATCCTGAA R : TTTCTGCCACCTCTTACATT	55	87
POU5F1	NM_001113060	F : AGTGAGAGGCAACCTGGAGA R : TCGTTGCGAATAGTCACTGC	60	166
BCL2L1	NM_214285.1	F : GGTTGACTTTCTCTCCTACAAG R : CTCAGTTCTGTTCTCTTCCAC	60	196
BIRC5	NM_214141	F : CCTGGCAGCTCTACCTCAAG R : GAAAGCACAACCGGATGAAT	60	233
CASP3	NM_214131	F : GAGGCAGACTTCTTGTATGC R : CATGGACACAATACATGGAA	55	236
FAS	AJ001202.1	F : GAGAGACAGAGGAAGACGAG R : CTGTTCAGCTGTATCTTTGG	54	194
BMP15	NM_001005155	F : CCTCGGGTACTACACTATG R : GGCTGGGCAATACATATCCT	60	192
CCNB1	NM_001113219	F : CCAACTGGTTGGTGTCACTG R : GCTCTCCGAAGAAAATGCAG	60	195
GDF9	XQ68750.1	F : GTCTCCAACAAGAGAGAGATTC R : CTGCCAGAAGAGTCATGTTAC	54	109
MOS	NM_001113219	F : TGGGAAGAAACTGGAGGACA R : TTCGGGTCAGCCCAGTTCA	60	121

F, forward; R: reverse.

2.8. Statistical analysis

Data from all experiments were analyzed using the general linear model procedure within the Statistical Analysis System software (SAS User's Guide 1985, Statistical Analysis System Inc., Cary,

NC, USA). The paired Tukey's multiple range test was used to determine significant differences. *P*-values <0.05 were considered significant.

3. RESULTS

3.1. ISO enhances the developmental rate of oxidative stress-exposed porcine oocytes during IVM

The effects of adding 0.02, 0.2, 2, and 20 μM ISO (0.02, 0.2, 2, and 20 ISO groups, respectively) to IVM medium containing (control group) or lacking (normal group) 200 μM H_2O_2 on the oocyte maturation efficiency were examined (Table 1). The percentage of surviving oocytes at the MII stage was significantly higher ($p < 0.05$) in the normal group than in the control, 0.02 ISO, 0.2 ISO, and 20 ISO groups, but did not significantly differ between the normal and 2 ISO groups. The percentage of surviving oocytes at the MII stage was significantly higher ($p < 0.05$) in the 2 ISO group than in the control, 0.02 ISO, and 20 ISO groups, but did not significantly differ between the normal, 0.2 ISO, and 2 ISO groups (normal, 84.0% ± 1.9%; control, 70.3% ± 2.9%; 0.02 ISO, 73.1% ± 2.2%; 0.2 ISO, 76.3% ± 2.4%; 2 ISO, 81.4% ± 2.8%; and 20 ISO, 74.6% ± 3.3%). After parthenogenetic activation (PA), the percentage of cleaved embryos on day 2 was significantly higher ($p < 0.05$) in the normal, 0.2 ISO, and 2 ISO groups than in the control, 0.02 ISO, and 20 ISO groups, but did not significantly differ between the normal, 0.2 ISO, and 2 ISO groups (normal, 60.7% ± 2.2%; control, 47.7% ± 1.9%; 0.02 ISO, 52.8% ± 2.7%; 0.2 ISO, 54.5% ± 1.3%; 2 ISO, 57.0% ± 3.3%; and 20 ISO, 49.9% ± 4.0%). The percentage of blastocyst formation on day 7 was significantly higher ($p < 0.05$) in the normal group than in the control, 0.02 ISO, and 20 ISO groups, but did not significantly differ between the normal and 2 ISO groups. The percentage of blastocyst formation on day 7 was significantly higher ($p < 0.05$) in the 0.2 ISO group than in the control, 0.02 ISO, and 20 ISO groups, but did not significantly differ between the 2 ISO group and the normal and 0.2 ISO groups (normal, 35.7% ± 1.8%; control, 23.8% ± 1.6%; 0.02 ISO, 26.3% ± 1.9%; 0.2 ISO, 28.1% ± 2.0; 2 ISO, 32.3% ± 2.0%; and 20 ISO, 25.1% ± 1.4%). Based on these results, 2 ISO was used in subsequent

experiments.

Table 2. Effect of different concentrations of isorhamnetin on *in vitro* maturation of porcine oocytes

Treatment group	H_2O_2 concentration (μM)	No. of GV-stage oocytes	No. (%) of		
			Surviving oocytes	Cleaved embryos on day 2	Blastocysts on day 7
Normal	0	350	294 (84.0±1.9)^{c}	178 (60.7±2.2)^{b}	64 (35.7±1.8)^{c}
Control	200	350	246 (70.3±2.9)^{a}	117 (47.7±1.9)^{a}	28 (23.8±1.6)^{a}
0.02 ISO	200	350	256 (73.1±2.2)^{a}	135 (52.8±2.7)^{a}	36 (26.3±1.9)^{a}
0.2 ISO	200	350	267 (76.3±2.4)^{b}	145 (54.5±1.3)^{ab}	41 (28.1±2.0)^{b}
2 ISO	200	350	285 (81.4±2.8)^{bc}	161 (57.0±3.3)^{b}	52 (32.3±2.0)^{bc}
20 ISO	200	350	261 (74.6±3.3)^{a}	128 (49.9±4.0)^{a}	32 (25.1±1.4)^{a}

GV, germinal vesicle; ISO, isorhamnetin. $^{a-c}p < 0.05$.

3.2. ISO elicits antioxidative effects during IVM of oxidative stress-exposed porcine oocytes

The intracellular ROS and glutathione (GSH) levels were measured to investigate the antioxidative effects of ISO during IVM of H_2O_2-exposed porcine oocytes (Fig. 1A). The fluorescence intensity of the ROS marker dichlorohydrofluorescein diacetate (DCFH-DA) was significantly lower ($p < 0.05$) in the normal group than in the control group, but did not significantly differ between the normal and 2 ISO groups (normal, 53.4 ± 1.6; control, 62.4 ± 1.7; and 2 ISO, 57.3 ± 2.2). The fluorescence intensity of the GSH marker CellTracker™ Blue 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CMF_2HC) was significantly higher ($p < 0.05$) in the normal and 2 ISO groups than in the control group, but did not significantly differ between the normal and 2 ISO groups (normal, 99.5 ± 3.0 pixels/oocyte; control, 79.3 ± 2.6 pixels/oocyte; and 2 ISO, 93.9 ± 3.0 pixels/oocyte). We investigated the effects of ISO on mRNA expression of the antioxidant-related genes nuclear factor erythroid 2-like 2 (*NFE2L2*), superoxide dismutase 1 (*SOD1*), and superoxide dismutase 2 (*SOD2*). The mRNA levels was normalized against those in the normal group (Fig. 1B). mRNA expression of *NFE2L2* and *SOD2* was significantly higher ($p < 0.05$) in the normal group than in the control and 2 ISO groups, and was significantly higher ($p < 0.05$) in the 2 ISO group than in the control group. mRNA expression of *SOD1* was significantly higher ($p < 0.05$) in the normal group than in the control and 2 ISO groups, but did not significantly differ between the control and 2 ISO groups.

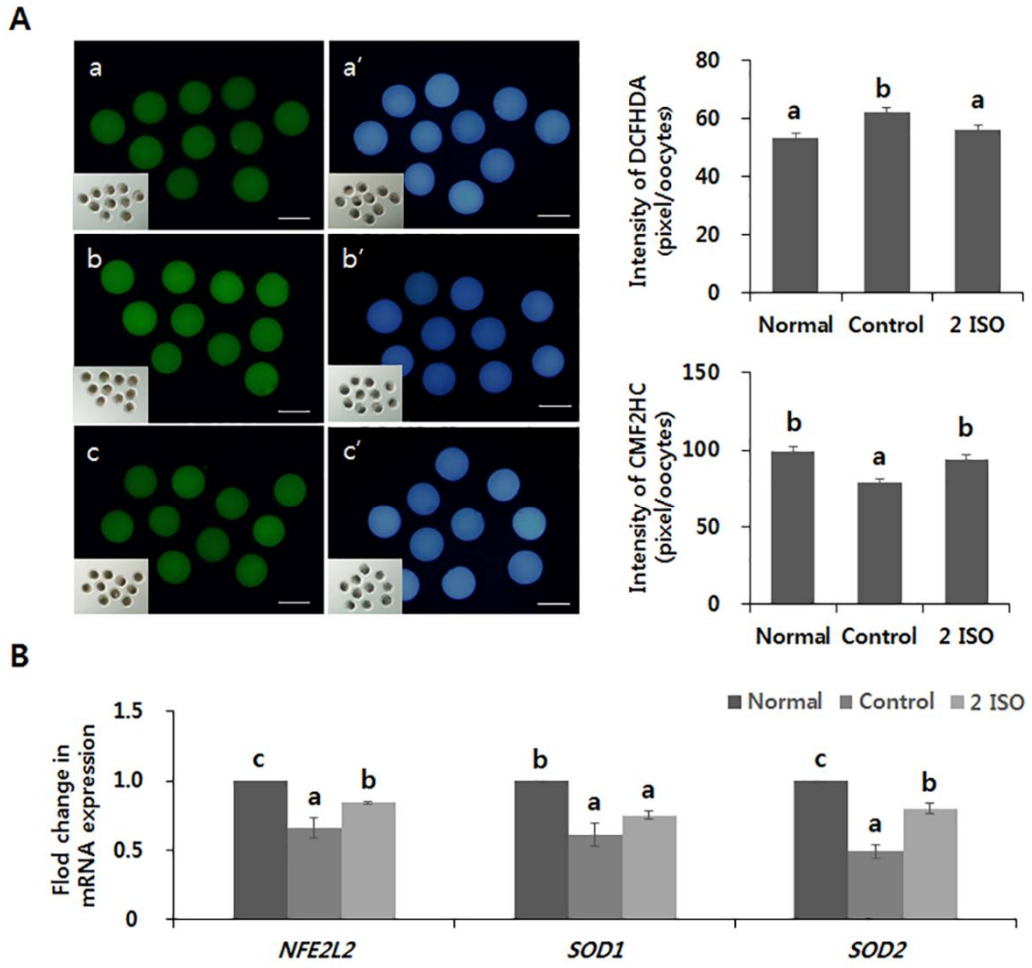

Fig. 1. Effect of ISO on the level of oxidative stress during IVM of porcine oocytes. H_2O_2 was used to induce oxidative stress. (A) Images of oocytes stained with DCFH-DA (green) and CellTracker™ Blue CMF_2HC (blue), and quantification of the fluorescence intensities of DCFH-DA and CellTracker™ Blue CMF_2HC. (a–c) ROS staining. (a'–c') GSH staining. (a) and (a') Normal group. (b) and (b') Control group. (c) and (c') 2 ISO group. Scale bar, 100 μm. (B) Relative mRNA expression of antioxidant-related genes (*NFE2L2*, *SOD1*, and *SOD2*). *ACTB* was used as an internal standard. Data were normalized against the corresponding levels in the normal group. Values are presented as mean ± SEM of X independent experiments ($^{a-c}p < 0.05$).

3.3. ISO enhances cumulus cell expansion, prevents chromosomal misalignment, and upregulates molecular maturation factors during IVM of oxidative stress-exposed porcine oocytes

We investigated the effect of ISO on cumulus cell expansion during IVM of H_2O_2-exposed porcine oocytes at the MII stage (Fig. 2). Microscopic analyses indicated that cumulus cell expansion was best in the normal group, and was better in the 2 ISO group than in the control group (Fig. 2A). We examined the effects of ISO on mRNA expression of the cumulus cell expansion-related genes cathepsin B (*CTSB*) and hyaluronan synthase 2 (*SHAS2*) at the MII stage. mRNA expression of *CTSB* was significantly higher ($p < 0.05$) in the normal and 2 ISO groups than in the control group, but did not significantly differ between the normal and 2 ISO groups. mRNA expression of *SHAS2* was significantly higher ($p < 0.05$) in the normal group than in the control and 2 ISO groups, and was significantly higher ($p < 0.05$) in the 2 ISO group than in the control group (Fig. 2B).

We next evaluated the effect of ISO on chromosomal alignment and spindle organization (Fig. 3). Oocytes were classified as abnormal or normal as described previously (Lenie, Cortvrindt, Eichenlaub-Ritter, & Smitz, 2008) (Fig. 3A). The percentage of oocytes with normal chromosomal alignment and spindle organization was significantly higher ($p < 0.05$) in the normal group than in the control and 2 ISO groups, and was significantly higher ($p < 0.05$) in the 2 ISO group than in the control group (normal, 78.2% ± 2.3%; control, 55.3% ± 3.4%; and 2 ISO, 68.4% ± 3.1%; Fig. 3B). To examine the effect of ISO on oocyte cytoplasmic maturation, we measured maternal gene expression and p44/42 mitogen-activated protein kinase (MAPK) activity (Fig. 4). mRNA expression of the maternal genes bone morphogenetic protein 15 (BMP15), cyclin B1 (CCNB1), growth differentiation factor-9 (GDF9), and serine/threonine kinase (MOS) was determined. mRNA expression of BMP15 and GDF9 was significantly higher ($p < 0.05$) in the normal and 2 ISO groups than in the control group, but did not significantly differ between the normal and 2 ISO groups. mRNA expression of CCNB1 was significantly higher ($p < 0.05$) in the 2 ISO group than in the

normal and control groups, and was significantly higher ($p < 0.05$) in the normal group than in the control group (Fig. 4A).

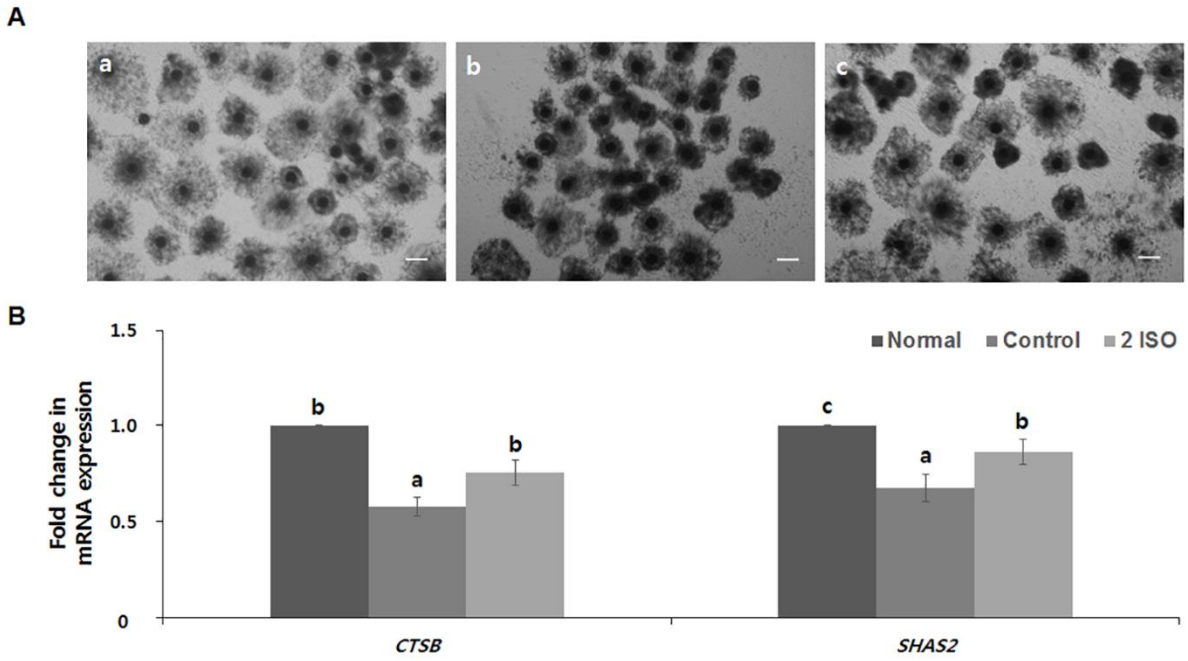

Fig. 2. Effect of ISO on cumulus cell expansion during IVM of porcine oocytes. (A) Degree of cumulus cell expansion after 44 h of IVM. (a) Normal group. (b) Control group. (c) 2 ISO group. Scale bar, 250 μm. (B) Relative mRNA expression of cumulus cell expansion-related genes (*CTSB* and *SHAS2*). *ACTB* was used as an internal standard. Data were normalized against the corresponding levels in the normal group. Values are presented as mean ± SEM of five independent experiments ($^{a\text{-}c}p < 0.05$).

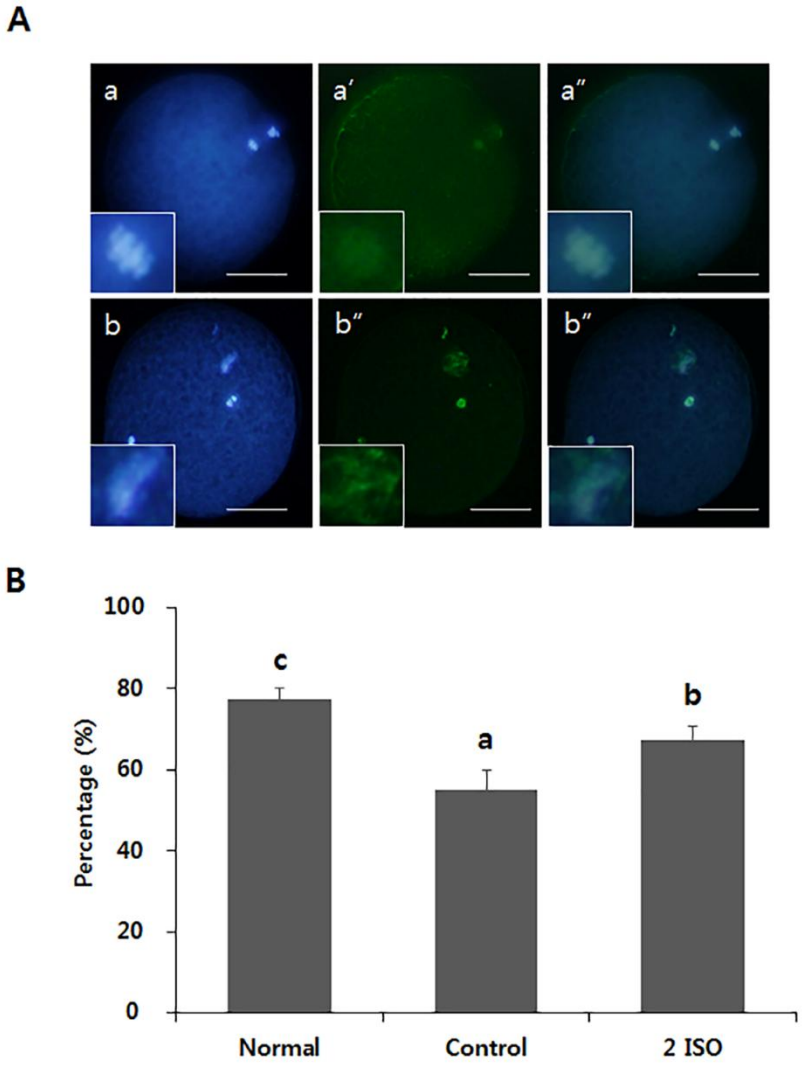

Fig. 3. Effect of ISO on spindle morphology during IVM of porcine oocytes. (A) Normal and abnormal chromosomal alignment and meiotic spindle formation in oocytes. (a) and (b) Nuclei of oocytes stained with Hoechst 33342. (a') and (b') Spindles of oocytes stained with an anti-α-tubulin antibody. (a'') and (b'') Merged images. (a–a'') Normal. (b–b'') Abnormal. Scale bar, 50 μm. (B) Percentage of oocytes with normal chromosomal alignment and meiotic spindle organization. Values are presented as mean ± SEM of five independent experiments ($^{a-c}p < 0.05$).

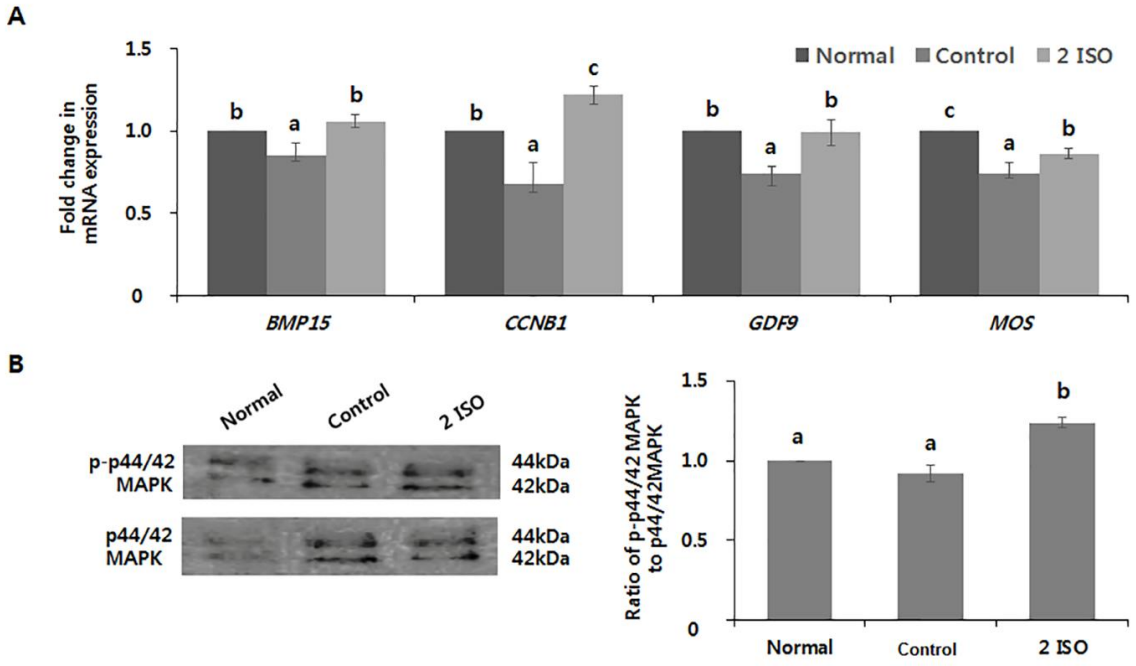

Fig. 4. Effect of ISO on cytoplasmic maturation during IVM of porcine oocytes. (A) Relative mRNA expression of maternal genes (*BMP15*, *CCNB1*, *GDF9*, and *MOS*). *ACTB* was used as an internal standard. Data were normalized against the corresponding levels in the normal group. (B) MAPK activity determined by western blot analysis and the ratio of phospho-p44/42 MAPK to p44/42 MAPK. Values are presented as mean ± SEM of four independent experiments ($^{a-c}p < 0.05$).

3.4. ISO improves the developmental capacity and quality of embryos derived from oxidative stress-exposed porcine oocytes

We studied the effect of ISO treatment of H_2O_2-exposed oocytes on blastocyst formation at day 7. The total cell number per blastocyst was significantly higher ($p < 0.05$) in the normal and 2 ISO groups than in the control group, but did not significantly differ between the normal and 2 ISO groups (normal, 82.9 ± 6.0; control, 60.2 ± 5.7; and 2 ISO, 76.6 ± 5.7; Fig. 5A). The percentage of apoptotic cells in blastocysts was significantly lower ($p < 0.05$) in the normal and 2 ISO groups than in the control group, but did not significantly differ between the normal and 2 ISO groups (normal, 3.6% ± 0.3%; control, 8.9% ± 1.3%; and 2 ISO, 4.9% ± 0.2%; Fig. 5B). We measured mRNA expression of the development-related genes sex-determining region Y-box 2 (*SOX2*), homeobox protein NANOG (*NANOG*), and POU domain, class 5, transcription factor 1 (*POU5F1*). mRNA expression of *SOX2* was significantly higher ($p < 0.05$) in the 2 ISO group than in the normal and control groups, but did not significantly differ between the normal and control groups. mRNA expression of *NANOG* and *POU5F1* was significantly higher ($p < 0.05$) in the 2 ISO group than in the normal and control groups, and was significantly higher ($p < 0.05$) in the normal group than in the control group. We measured mRNA expression of the anti-apoptotic genes B-cell lymphoma 2-like 1 (*BCL2L1*) and baculoviral IAP repeat-containing 5 (*BIRC5*). mRNA expression of *BCL2L1* was significantly higher ($p < 0.05$) in the 2 ISO group than in the normal and control groups, and was significantly higher ($p < 0.05$) in the normal group than in the control group. mRNA expression of *BIRC5* was significantly higher ($p < 0.05$) in the normal and 2 ISO groups than in the control group, but did not significantly differ between the normal and 2 ISO groups. We measured mRNA expression of the pro-apoptotic genes cysteine-aspartic acid protease 3 (*CASP3*) and Fas cell surface death receptor (*FAS*). mRNA expression of *CASP3* and *FAS* was significantly lower ($p < 0.05$) in the normal group than in the control and 2 ISO groups, and was significantly lower ($p < 0.05$) in the 2 ISO group than in the control group.

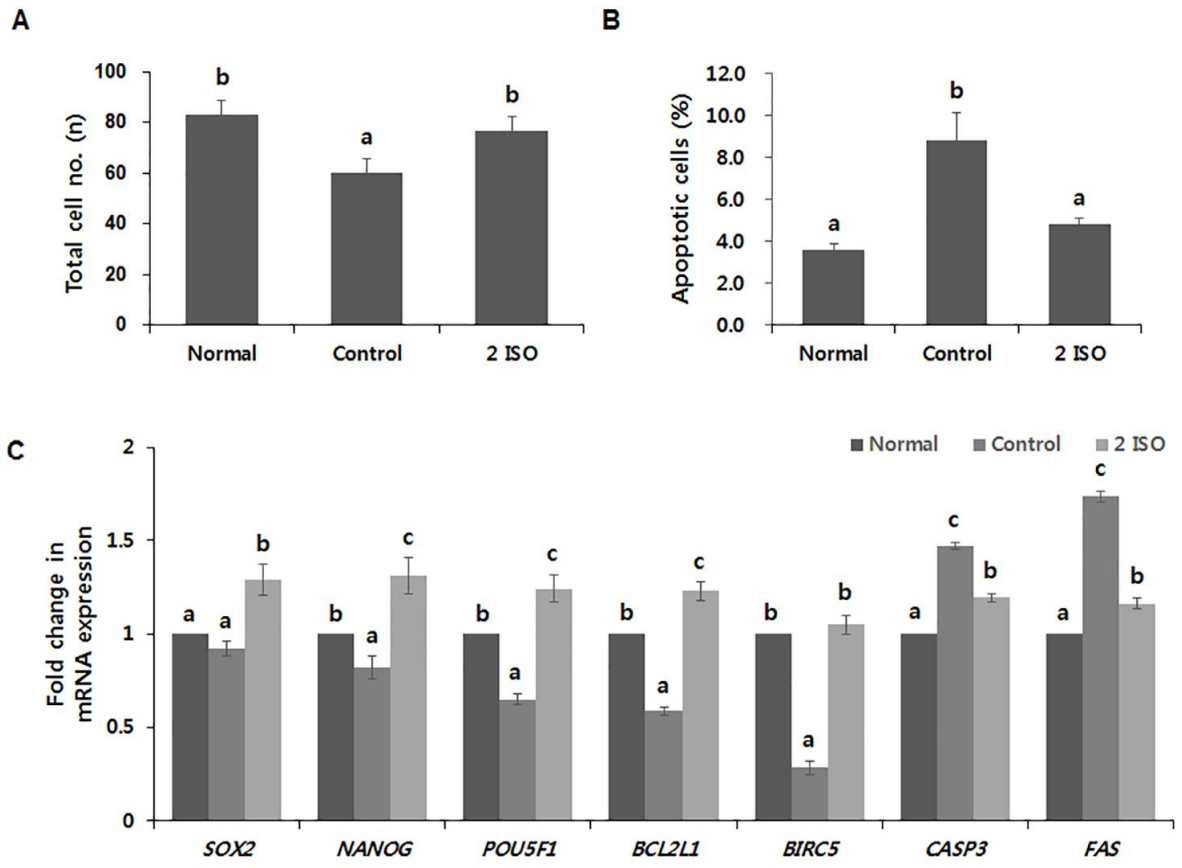

Fig. 5. Effect of ISO treatment during IVM of porcine oocytes on subsequent embryo development. (A) Total cell number per blastocyst. (B) Percentage of apoptotic cells in blastocysts. (C) Relative mRNA expression of development-related (*SOX2*, *NANOG*, and *POU5F*), anti-apoptotic (*BCL2L1* and *BIRC5*), and pro-apoptotic (*CASP3* and *FAS*) genes. *ACTB* was used as an internal standard. Data were normalized against the corresponding levels in the normal group. Values are presented as mean ± SEM of seven independent experiments ($^{a-c}p < 0.05$).

4. DISSCUSION

This study investigated the effect of ISO on IVM of oxidative stress-exposed porcine oocytes and the developmental capacity of embryos derived from these oocytes. ISO increased the GSH level, enhanced cumulus cell expansion, prevented chromosomal misalignment, activated MAPK in porcine oocytes, and increased the total cell number and decreased the percentage of apoptotic cells in blastocysts derived from these oocytes.

Porcine oocytes were matured in vitro for 44 h in the presence of 200 μM H2O2 and 0, 0.02, 0.2, 2, or 20 ISO, or in the absence of ISO and H2O2. However, in vitro-matured oocytes have a variable lack compared with in vivo matured oocytes because the oxygen concentration is higher in vitro than in vivo (Tatemoto et al., 2000). Great efforts have been made to improve IVM efficiency (Yoshida, Ishigaki, & Pursel, 1992) which include changing the culture medium composition (Marques et al., 2007), altering the atmospheric composition (Kang et al., 2012) supplying hormones (Silvestre et al., 2007), and adding antioxidants (Hennings et al., 2016). Addition of antioxidants to culture media reduces oxidative stress (Tatemoto, Ootaki, Shigeta, & Muto, 2001). We demonstrated that the percentages of surviving oocytes, cleaved embryos, and blastocysts formation were increased in the 2 ISO group (Table 1). ISO has a high DPPH-scavenging ability dependent on its phenolic hydroxyl group (Zuo et al., 2011), and it improves the viability of oxidative stress-exposed retinal pigment epithelial cells by activating PI3K/Akt. The PI3K/Akt signaling pathway is fundamental in animals, and functions in cell growth, proliferation, survival, migration, metabolism, and apoptosis (Knowles, Platt, Ross, & Hurst, 2009). Therefore, we demonstrate that ISO enhances the development rate of oxidative stress-exposed porcine oocytes, and use 2 ISO in subsequent experiments.

Intracellular oxidative and antioxidative activities are primarily determined by ROS and GSH, respectively. ROS naturally form during metabolic processes (Storz & Imlay, 1999), but external oxygen and an inefficient antioxidant system increase ROS generation in vitro (Armand et al., 2019).

An excessively high ROS level directly or indirectly interferes with mitochondrial function (Zhang & Liu, 2002). On the other hand, GSH is a cofactor of glutathione peroxidase and glutathione-S-transferase, and plays an important role in protecting cellular lipids, proteins, and nucleic acids against oxidative stress (Gerard-Monnier & Chaudiere, 1996).This study showed that treatment with 2 ISO altered the ROS and GSH levels (Fig. 1A), and increased mRNA expression of the antioxidant-related genes NFE2L2 and SOD2 (Fig. 1B). NFE2L maintains mitochondrial redox homeostasis by upregulating antioxidant genes (Ryoo & Kwak, 2018) and plays an important role in defense against oxidative stress (Zelko, Mariani, & Folz, 2002). Activated NFE2L promotes expression of the antioxidants SOD1 and SOD2 (Dong, Sulik, & Chen, 2008). SOD is the first line of defense against superoxide produced as a byproduct of oxidative phosphorylation (Li et al., 1995). ISO increases the total level of NFE2L2 in mouse-derived C2C12 myoblasts by activating the Nrf2/HO-1 pathway, which involves activation of the ERK pathway (Y. H. Choi, 2016), and it enhances SOD activity in the mouse hippocampus and prefrontal cortex (Ishola, Osele, Chijioke, & Adeyemi, 2019). ISO acts as an antioxidant by scavenging DPPH, donating electrons to reactive free radicals, and chelating iron (Pengfei et al., 2009). It functions as an antioxidant in human cells by activating p38-MAPK (Bao & Lou, 2006). In summary, ISO protects cells against oxidative stress by acting as an antioxidant, thereby reducing the ROS level and increasing the GSH level.

This study investigated the effect of ISO on cumulus cell expansion, chromosomal alignment, and molecular maturation factors in porcine oocytes. Cumulus cells surround the oocyte and play a important role in processes of oocyte maturation and fertilization. These cells expand and surround oocytes after ovulation (Downs, Daniel, Bornslaeger, Hoppe, & Eppig, 1989). At the time of ovulation, cumulus cells have progressed to MII (Gerard-Monnier & Chaudiere, 1996). During cumulus cell expansion, an extracellular matrix consisting of hyaluronic acid (HA), proteoglycans, and proteins accumulates in the intercellular space and induces oocyte maturation (Russell & Salustri, 2006). In the present study, treatment with 2 ISO enhanced cumulus cell expansion and expression of the cumulus expansion-related gene SHAS2 (Fig. 2). SHAS2 stimulates production and extrusion of HA (Itano et al., 1999), and its expression increases during cumulus cell expansion (Fulop, Salustri, &

Hascall, 1997). The cumulus cell expansion-related gene CTSB plays a regulatory role in cell death via caspase-3 stimulation in oocytes (Eykelbosh & Van Der Kraak, 2010), and its upregulation in cumulus cells indicates that oocytes have a low developmental competence (Bettegowda et al., 2008). In summary, ISO protects cumulus cells against oxidative stress and improves the quality of cumulus-porcine oocyte complexes under oxidative stress. Interestingly, the present study showed that ISO increased the percentage of oocytes with normal chromosomal alignment and spindle organization (Fig. 3B), elevated MAPK activity, and upregulated maternal gene expression (Fig. 4). The meiotic spindle comprises microtubules and is crucial for normal chromosomal alignment and separation of maternal chromosomes during MI and MII in oocytes (Liu, Sun, Li, Jiao, & Wang, 2003). It is essential for the maintenance of chromosomal organization and formation of the second polar body (Schatten, Simerly, & Schatten, 1985). Oxidative stress increases abnormal spindle alignment (W. J. Choi et al., 2007), and spindle disruption promotes chromosomal misalignment (Eroglu, Toth, & Toner, 1998). MAPK, which regulates cell cycle progression by modulating microtubules and actin filaments, is an essential regulator of oocyte maturation (Q. Y. Sun et al., 2002), but its activity decreases upon oxidative stress (Inoue et al., 2005). MAPK is activated at the germinal vesicle breakdown stage, localizes to the cytoplasm and around chromosomes from MI to MII, and is essential for resuming meiosis in MII and maintaining arrest (Villa-Diaz & Miyano, 2004). MAPK is activated by MOS protein, an active component of cytostatic factor, which is responsible for meiotic arrest at MII (Newman & Dai, 1996). GDF9 and BMP15 play an important role in the regulation of fertility (Juengel et al., 2004) and regulate oogenesis by interacting with each other (Hussein, Thompson, & Gilchrist, 2006). CCNB is an important meiotic regulator and forms a complex with cyclin-dependent kinase 1, which permits transition from GII to M phase (Robert, Hue, McGraw, Gagne, & Sirard, 2002). Therefore, upregulation of these genes is beneficial in oxidative stress-exposed porcine oocytes. A recent study reported that ISO inhibits proliferation of cells arrested in GII/M phase and promotes formation of cytoplasmic vacuoles, which are indicative of apoptotic cell death mediated by ROS and the ERK signaling pathway (Chen et al., 2020). In summary, we demonstrate that ISO protects the nucleus and regulates maturation factors in oxidative

stress-exposed porcine oocytes. Collectively, these results indicate that ISO improves porcine oocyte maturation under oxidative conditions.

To further investigate whether ISO affects subsequent embryo development, oocytes were parthenogenetically activated and cultured in vitro for 7 days. Treatment with 2 ISO increased the total cell number per blastocyst and reduced the percentage of apoptotic cells (Fig. 5A and B), and regulated development- and apoptosis-related genes (Fig. 5C). Apoptosis is associated with cellular stress and cell death (Mizushima, 2007). The apoptosis supports embryo survival under healthy conditions by selectively eliminating abnormal cells with nuclear and chromosomal abnormalities, which may lead to the loss of the whole embryo during early embryonic development (J. Choi et al., 2008). It is important that the tendency for the apoptosis is consistently balanced, because high percentage of apoptotic cell inhibits embryonic development (Kwak et al., 2012). In a recent study, resveratrol supplementation was downregulating apoptosis-related genes and had better developmental competence in porcine oocyte (Kwak et al., 2012). Another study found that BME modulates GSH level and raises total cell number in blastocysts derived from matured porcine oocytes (Abeydeera, Wang, Cantley, Prather, & Day, 1998). The cell number have been used to determine embryo viability and increased cell numbers have been associated with advanced development of embryo (Papaioannou & Ebert, 1988). We examined expression of development-related (SOX2, NANOG, and POU5F1) and apoptosis-related (BCL2L1, BIRC5, CASP3, and FAS) genes. Recent studies showed that SOX2, NANOG, and POU5F1 contribute to activation of zygotic genes during the maternal-to-zygote transition (Lee et al., 2013), while POU5F1 and SOX2 function in all the main embryonic regulatory pathways (Leichsenring, Maes, Mossner, Driever, & Onichtchouk, 2013). Anti-apoptotic members of the BLC-2 family regulate pro-apoptotic proteins (Marques et al., 2007). BIRC5 belongs to the inhibitor of apoptosis family and is involved in regulation of the mitotic spindle, especially at G2/M phase. BIRC5 protein is expressed during embryonic and fetal development (Ambrosini, Adida, & Altieri, 1997). FAS activates caspase-3 by stimulating denitrosylation of thiol in its active site (Mannick et al., 1999). Our results showed that ISO improves the developmental capacity and quality of embryos under oxidative conditions.

In conclusion, this study demonstrates that the antioxidant ISO effectively protects oocytes against oxidative stress by reducing the level of ROS and thereby minimizes the deterioration in oocyte quality, leading to subsequent good-quality embryonic development. Thus, ISO may be a good supplement during porcine oocyte maturation to improve the efficiencies of in vitro fertilization, intracytoplasmic sperm injection, and somatic cell nuclear transfer.

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ABSTRACT KOREAN

플라보노이드 화합물 이소람네틴은 케르세틴의 3' 메톡실 유도체이며 항산화, 항염증 및 항암작용을 비롯한 다양한 생물학적 작용을 한다. 본 연구는 산화적 스트레스에 노출된 돼지 난모세포의 체외 성숙 동안 ROS 수준, 효율성, 난구세포의 확장을 조절하여 배아의 발육능력에 어떤 영향을 미치는지 조사하였다. 돼지의 난소로부터 회수된 미성숙 난자는 이소람네틴을 포함한 배양액에 44시간 체외성숙 하였다. 실험의 결과, 난모세포에 2μM의 이소람네틴(2 ISO) 농도가 다른 농도 0.02μM (0.02 ISO), 0.2μM (0.2 ISO), 20μM (20 ISO)의 이소람네틴보다 배아형성률이 높고 대조군과 비슷한 수준을 보였다. 그리고 GSH 수준과 mRNA 발현 (NFE2L2 및 SOD2)은 2 ISO에서 증가했고 ROS 수준은 감소했다. 2 ISO를 처리하면 난구세포 확장 관련 유전자 (SHAS2)의 mRNA 발현을 증가시키고 염색체 정렬의 불량을 억제하며, MAPK 활성이 2 ISO에서 증가되었다. 또한 2 ISO에서 각각 배반포 당 총 세포 수가 증가하고 세포사멸을 감소시켰다. 이러한 결과는 2 ISO가 난모세포의 체외성숙 동안 산화적 스트레스로부터 돼지 난모세포를 보호하여 배아 발달을 향상시킨다는 것을 입증한다. 따라서, 우리는 이소람네틴이 돼지 난모세포의 체외성숙에 유용한 보충제라는 것을 제안한다.

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