A Dissertation for the Degree of Master of Science

Study on β-Cryptoxanthin as Antioxidant in the Potential of Porcine Follicular Oocytes and Subsequent Embryo Development

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Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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ABSTRACT

Oxidative stress is mainly responsible for the poor quality of *in vitro* matured oocytes. To overcome this problem, this study investigated the effects of the antioxidant β-cryptoxanthin, a kind of antioxidants, on *in vitro* maturation (IVM) of porcine oocytes and *in vitro* development of following embryos. The immature oocytes collected from porcine ovaries were matured in IVM medium containing 0, 0.1, 1, 10 and 100 μM of β-cryptoxanthin (Control, 0.1 B, 1 B, 10 B and 100 B). As a result of this experiment, the rate of polar emission after 44 h in vitro maturation was increased in the 1 B group. And in the 1 B treatment group, expression of the maturation related genes was significantly improved in both of cumulus cells and oocytes. During whole maturation periods, this treatment also significantly decreased and increased the levels of reactive oxygen species and glutathione, respectively. Similarly, expression of the antioxidant genes superoxide dismutase 1 and peroxiredoxin 5 was increased in the 1 B group. After parthenogenetic activation, the cleavage rate did not differ between the control and 1 B groups; however, the blastocyst formation rate was higher in the latter group than in the former group. The total cell number per blastocyst and relative mRNA levels of pluripotency marker and antioxidant genes were significantly higher in the 1 B group than in the control group. These results demonstrate that β-cryptoxanthin decreases oxidative stress in porcine oocytes and improves their quality and developmental potential.

Keyword: *In vitro maturation, Oocyte, Oxidative stress, Antioxidant, Porcine*

1. INTRODUCTION

In vitro maturation (IVM) is fundamental for *in vitro* production of embryos. Once matured, oocytes can be fertilized by sperm. This process involves nuclear and cytoplasmic maturation. Specifically, the cell cycle progresses to metaphase of the second meiotic division (MII), and organelles are rearranged, the cytoskeleton is repositioned, and levels of specific proteins are altered in the cytoplasm (Heikinheimo and Gibbons, 1998; Setti AS et al., 2011). Abnormal oocyte maturation perturbs embryo development. The efficiency of oocyte maturation is lower *in vitro* than *in vivo*; therefore, many researchers have sought to improve *in vitro* culture systems in an attempt to produce high quality embryos.

Oxidative stress, one of the most documented causes of damage to *in vitro*-produced embryos, is triggered by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), and the hydroxyl radical ($^{\bullet}OH$) (Slimen et al., 2014). When present in excess, ROS react with and damage nucleic acids, proteins, lipids, and organelles. This leads to abnormal oocyte maturation and decreases the developmental competence of embryos. Large amounts of ROS are generated during oocyte maturation. Antioxidative systems in oocytes and peripheral reproductive organs minimize oxidative damage by removing ROS *in vivo* (Guerin et al., 2001). However, in oocytes matured *in vitro*, the production of ROS is increased and the ROS concentration remains high due to their inefficient removal. These ROS damage oocytes and thereby decrease the developmental competence (Goto et al., 1993). Therefore, to improve the production efficiency and quality of oocytes and embryos *in vitro*, it is important to maintain a physiological level of intracellular ROS. Accordingly, many studies have sought to develop *in vitro* culture methods that reduce ROS generation or more efficiently eliminate ROS. Various conditions are useful in this regard, including a low oxygen concentration (Hashimoto et al., 2000); supplementation of enzymatic antioxidants

such as superoxide dismutase (SOD), catalase, and peroxiredoxin (Alvarez et al., 2014; Fakruzzaman et al., 2015); and addition of non-enzymatic antioxidants such as flavonoids (Kang et al., 2016), cysteamine (Kobayashiet al., 2006), anthocyanin (You et al., 2010), and melatonin (Gao et al., 2012).

β-cryptoxanthin is important for human life and is the main vitamin present in citrus fruits, one of the most highly produced and consumed types of fruit in the world. This vitamin is one of the six major carotenoids, along with β-carotene, lycopene, lutein, zeaxanthin, and α-carotene, in human serum. β-cryptoxanthin has potent anti-cancer effects (Gallicchio et al., 2008) and antioxidative activities such as scavenging free radicals (Miller et al., 1996 and Fu et al., 2010) and suppressing lipid peroxidation (Katsuura et al., 2009) and nitrogen oxide production (Murakami et al., 2000).

This study investigated the effects of β-cryptoxanthin on IVM of porcine oocytes and the developmental competence of embryos derived from these oocytes. We examined the maturation efficiency, level of oxidative stress, cumulus cell expansion, and expression of maturation-related factors during oocyte maturation in addition to embryo developmental competence and blastocyst quality. Our results suggest that β-cryptoxanthin can enhance the developmental competence and quality of *in vitro*-produced embryos, and may thus improve assisted reproductive technology (ART) and the production of cloned animals.

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

Pre-pubertal 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℃. 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 h at 38.8℃ in 5% CO_2 and 95% air. The media was supplemented with 0, 0.1, 1, 10, or 100 μM β-cryptoxanthin (Extrasynthese, Genay, France).

2.3. Parthenogenetic activation (PA) and embryo culture

After 44 h of maturation, cumulus cells were removed from COCs by pipetting in the presence of 0.1% (w/v) hyaluronidase. Denuded oocytes were parthenogenetically activated by treatment with 5 μM Ca^{2+}-ionomycin for 5 min. Activated oocytes were cultured in

porcine zygote medium (PZM)-5 supplemented with 7.5 μg/mL cytochalasin B for 3 h, transferred to PZM-5 containing 0.4% (w/v) BSA, and cultured for 7 days at 38.8℃ in a humidified atmosphere of 5% CO_2 and 95% air.

2.4. Measurement of intracellular ROS and glutathione (GSH) levels

Dichlorohydrofluorescein diacetate (DCHFDA) and CellTracker™ Blue 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (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 DCHFDA or 100 μM CellTracker™ Blue CMF_2HC in the dark for 20 min at 38.8℃. 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. The excitation and emission spectra of CellTracker™ Blue CMF_2HC dye 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 Image J software (NIH, Bethesda, MD, USA). Background fluorescence values were subtracted from the final values before statistical analysis. The experiment was independently repeated three times, with 10–20 oocytes per experiment.

2.5. Hoechst staining

Blastocysts were cultured for 7 days after PA, fixed overnight at 4℃ in 4.0% (w/v)

paraformaldehyde prepared in phosphate-buffered saline (PBS), washed more than three times with PBS containing 0.1% BSA, and incubated with Hoechst 33342 (1 μg/mL) at 38.8℃ 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 three times, and at least ten blastocysts were examined per group.

2.6. mRNA extraction and cDNA synthesis

mRNA was isolated from more than three biological replicates, with 30–40 oocytes per replicate, using the Dynabeads mRNA Direct kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. mRNA from each sample was collected in 10 μL elution buffer from 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.

2.7. Real-time reverse transcription (RT)-PCR

The protocol used was basically the same as that described previously (Lee et al., 2015). Real-time RT-PCR was performed using the primer sets listed in Table 1 and a StepOne Plus 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℃, followed by 39 cycles of 15 sec at 95℃ and 60 sec at 54℃ or 60℃. Samples were then cooled to 12℃. Relative gene expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) after normalization against the expression level of a housekeeping gene (GAPDH or β-actin). The experiment was

independently repeated three times.

Table 1. Primers used for real-time PCR

Gene	GenBank accession no.	Primer sequence*	Annealing temp (℃)	Product size (bp)
GAPDH	AF017079	F: GGGCATGAACCATGAGAAGT R: AAGCAGGGATGATGTTCTGG	54	230
SOD1	GU944822.1	F: GCCACTGTGTACATCGAAGAT R: GTGATCCCAATTACACCACAG	54	173
PRDX5	AF110735.2	F: GGCATGTCTGAGTGTTAATGAC R: CAAAGAGAGACACCAAGGAATC	54	152
RARG	U82629.1	F: CTCATGAAGATTACCGACCT R: GAATCTCCATCTTCAGGGTA	54	76
RXRA	AF103947.1	F: ATGAAGCGTGAAGCCGTGCAGG R: TTCTCCACCGGCATGTCCTCGTT	54	104
SHAS2	NM_214053.1	F: ACTGTCCAGTTAGTAGGTCTCA R: ACATGTACAACACCGAGTAGAG	54	100
HAPLN1	NM_001004028.1	F: ACCTTACCCTGGAGGATTAT R: CTAATGCTACCACAGCTGTATC	54	81
CTSB	NM_001097458.1	F: CTCTAGGAACGAGAAGGAGAT R: CCAGACTTATACTGCAGGAAG	54	99
BMP15	NM_001005155	F: CCCTCGGGTACTACACTATG R: GGCTGGGCAATCATATCCT	60	192
CCNB1	L48205	F: TTGACTGGCTAGTGCAGGTT R: CTGGAGGGTACATTTCTTCA	60	177
MOS	NM_001113219	F: TGGGAAGAAACTGGAGGACA R: TTCGGGTCAGCCCAGTTCA	60	121
Pou5f1	NM_001113060	F: AGTGAGAGGCAACCTGGAGA R: GTAGTAGGCTCTGGGGTAATACT	60	166
CDX2	AM778830	F: AGCCAAGTGAAAACCAGGAC R: TGCGGTTCTGAAACCAGATT	60	178

*F, forward; R, reverse.

2.8. Western blot analysis

The protocol was basically the same as that described previously (Lee et al., 2015). 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℃. Proteins were resolved on 5–12% Tris SDS-PAGE gels for 1.5 h at 80–100 V. Samples were then transferred to Hybond-ECL nitrocellulose membranes (Amersham, Buckinghamshire, UK) at 300 mA for 2 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 mitogen-activated protein kinase (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 Tris-buffered saline containing Tween-20 (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 three times.

2.9. 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 Student's t-test was used to compare relative gene expression. P values <0.05 were considered significant.

3. Results

3.1. β-cryptoxanthin treatment enhances IVM of porcine oocytes

We compared the maturation efficiency of oocytes cultured in IVM medium containing 0, 0.1, 1, 10, and 100 μM β-cryptoxanthin, which were referred to as the control, 0.1 B, 1 B, 10 B, and 100 B groups, respectively. The oocyte maturation efficiency was determined by quantifying the polar body (PB) emission rate after 44 h of IVM (Table 2). The rate of PB emission was significantly higher in the 1 B group than in the control and 100 B groups (control, 50.1 ± 3.59%; 1 B, 60.1 ± 4.03%, and 100 B, 46.5 ± 3.90%; $P<0.05$). However, it did not significantly differ between the other β-cryptoxanthin-treated groups and the control group (control, 50.1 ± 3.59%; 0.1 B, 55.1 ± 4.37%; 10 B, 50.7 ± 4.94%, and 100 B, 46.5 ± 3.90%).

Table 2. Effect of β-cryptoxanthin treatment on polar body emission during *in vitro* maturation of porcine oocytes

Group	Polar body emission (%)
Control	50.1 ± 3.59 [a]
0.1 B	55.1 ± 4.37 [ab]
1 B	60.1 ± 4.03 [b]
10 B	50.7 ± 4.94 [ab]
100 B	46.5 ± 3.90 [a]

Values with different superscript letters are significantly different ($p<0.05$).

3.2. β-cryptoxanthin treatment during IVM influences cumulus cell expansion and expression of maturation factors in COCs

To investigate the effect of β-cryptoxanthin on expansion of cumulus cells, we analyzed expression of the cumulus cell expansion-related genes hyaluronan synthase 2 (*SHAS2*), hyaluronan and proteoglycan link protein 1 (*HAPLN1*), and cathepsin B (*CTSB*) in cumulus cells obtained from COCs at MII. Expression of *SHAS2* and *HAPLN1* was significantly higher in the 1 B group than in the control group (Fig. 1A, $P<0.01$). By contrast, expression of *CTSB* was significantly lower in the 1 B group than in the control group (Fig. 1A, $P<0.01$).

To investigate the effect of β-cryptoxanthin on porcine oocyte maturation at the genetic level, we analyzed expression of the maternal genes bone morphogenetic protein 15 (*BMP15*), cyclin B1 (*CCNB1*), and the proto-oncogene *MOS*, which encodes a serine/threonine kinase, as well as MAPK activity. Expression of *BMP15*, *CCNB1*, and *MOS* was significantly higher in the 1 B group than in the control group (Fig. 1B, $P<0.05$). Active phospho-p44/42 MAPK migrated as a doublet in lysates of maturing porcine oocytes (Fig. 1C). The ratio of phospho-p44/42 MAPK to p44/42 MAPK was significantly higher in the 1 B group than in the control group (Fig. 1C; control, 41.9 ± 0.6% and 1 B, 54.8 ± 5.6%; $P<0.05$).

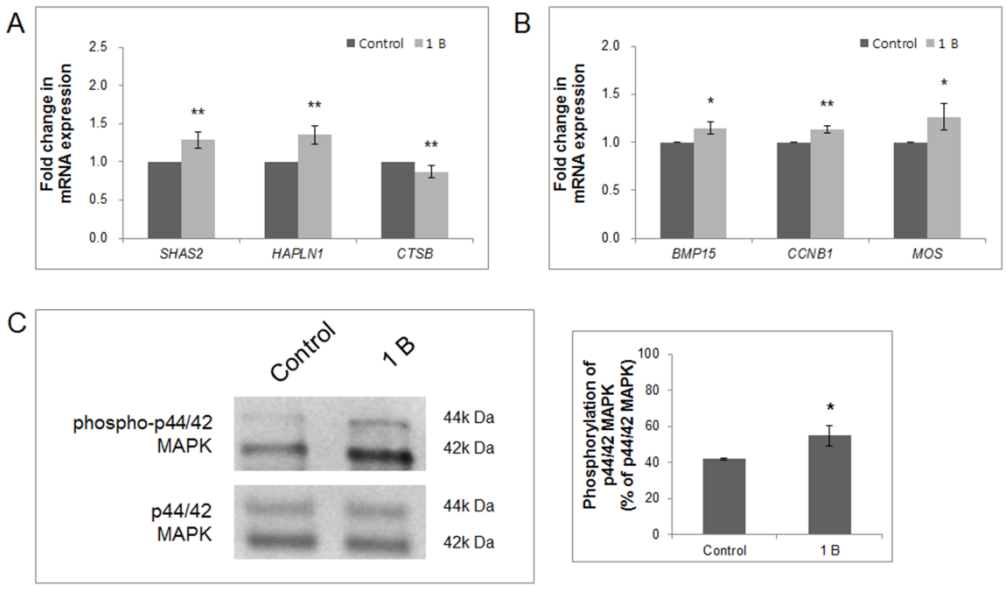

Fig. 1. Effect of β-cryptoxanthin treatment on cumulus cell expansion and oocyte maturation. Relative expression of cumulus cell expansion-related genes in cumulus cells at the MII stage that had been separated from COCs matured in the presence or absence of 1 μM β-cryptoxanthin (A). Expression of maternal genes (B) and expression of phospho-p44/42 MAPK and the ratio of phospho-p44/42 MAPK to p44/42 MAPK (C) in control and 1 μM β-cryptoxanthin-treated oocytes. GAPDH was used as an internal standard. Significant differences compared with control oocytes are indicated (*$p<0.05$ and **$p<0.01$). Values represent means ± standard errors of the mean of independent experiments.

3.3. Supplementation of IVM media with β-cryptoxanthin has an antioxidative effect during porcine oocyte maturation

To assess the antioxidative effect of β-cryptoxanthin during IVM of porcine oocytes, the intracellular levels of ROS and GSH were determined at the germinal vesicle breakdown (GVBD, 26 h), metaphase I (MI, 32 h), and MII (44 h) stages (Fig. 2A and 2B). At each stage, the fluorescence intensity of ROS labeling was significantly lower in the 1 B group than in the control group (Fig. 2A, $P<0.05$). Moreover, the fluorescence intensity of GSH labeling was significantly higher in the 1 B group than in the control group at all stages (Fig. 2B, $P<0.05$).

Additionally, we examined the effect of β-cryptoxanthin on expression of the antioxidant genes *SOD1* (Fig. 2Ca) and peroxiredoxin 5 (*PRDX5*, Fig. 2Cb). The mRNA levels were determined at each stage and normalized by that in the control group. mRNA expression of these genes was significantly higher in the 1 B group than in the control group at all stages ($P<0.01$).

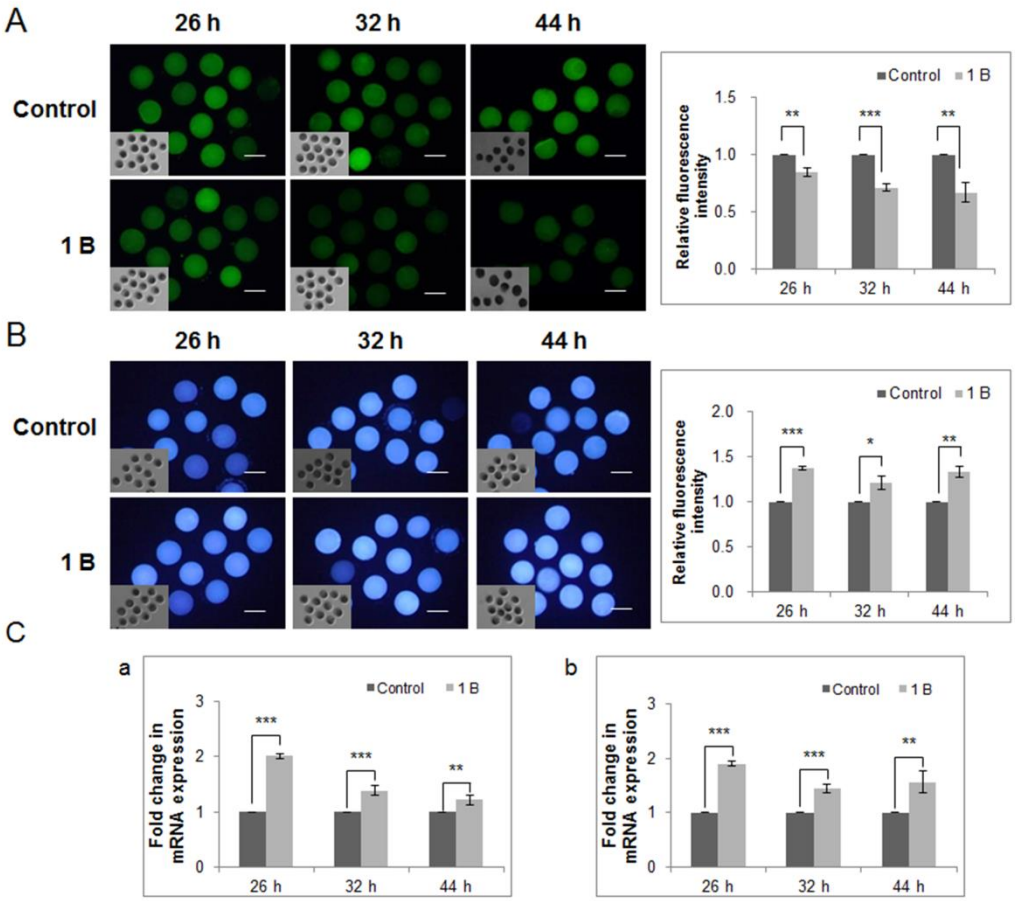

Fig. 2. Effect of β-cryptoxanthin treatment on the level of oxidative stress during porcine oocyte maturation. Intracellular ROS were detected using DCHFDA (A, green) and intracellular GSH was detected using CellTracker™ Blue CMF_2HC (B, blue). The fluorescence intensities were quantified according to the maturation stage. Bar = 120 μm. The time points correspond to GVBD (26 h), MI (32 h), and MII (44 h). Relative mRNA expression of the antioxidant genes *SOD1* (a) and *PRDX5* (b) in oocytes matured for 44 h in the presence or absence of 1 μM β-cryptoxanthin (C). GAPDH was used as an internal standard. Significant differences compared with control oocytes are indicated (*$p<0.05$, **$p<0.01$, and ***$p<0.001$). Values represent means ± standard errors of the mean of independent experiments.

3.4. β-cryptoxanthin is converted into an active form in cumulus cells and oocytes

β-cryptoxanthin is converted into retinoic acid, which binds to the retinoid receptor; therefore, we examined expression of the retinoid receptor genes retinoic acid receptor gamma (*RARG*) and retinoid X receptor alpha (*RXRA*) in cumulus cells (Fig. 3A) and oocytes (Fig. 3B) at each stage. In cumulus cells, expression of both genes was significantly higher in the 1 B group than in the control group at the GVBD stage (*RARG*, $p<0.05$ and *RXRA*, $P<0.01$). At the MI and MII stages, expression of both genes was significantly lower in the 1 B group than in the control group ($P<0.001$). In oocytes, expression of *RARG* and *RXRA* was significantly higher in the 1 B group than in the control group at all stages ($P<0.05$), with the exception of *RARG* expression at the GVBD stage, which did not significantly differ between the two groups.

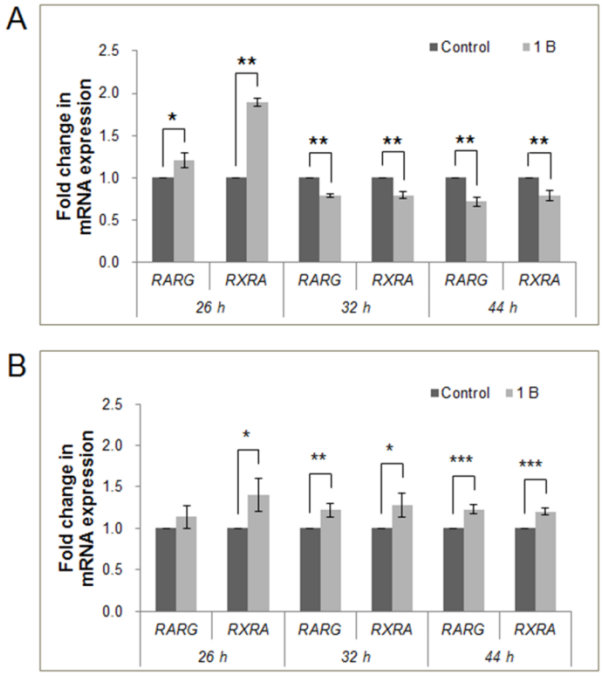

Fig. 3. Effect of β-cryptoxanthin treatment on relative mRNA expression of *RARG* and *RXRA*. *RARG* and *RXRA* expression in cumulus cells (A) and oocytes (B) according to the maturation stage. GAPDH was used as an internal standard. Significant differences compared with the control group are indicated (*$p<0.05$, **$p<0.01$, and ***$p<0.001$). Values represent means ± standard errors of the mean of independent experiments.

3.4. β-cryptoxanthin treatment during IVM improves the developmental potential and quality of parthenogenetically activated embryos

To investigate whether β-cryptoxanthin treatment during IVM influences the development and quality of embryos, oocytes were matured in the presence or absence of 1 μM β-cryptoxanthin and then parthenogenetically activated. The cleavage and blastocyst formation rates were calculated at 2 and 7 days after PA, respectively. Furthermore, the total cell number per blastocyst and the relative expression of pluripotency marker and antioxidant genes in blastocysts were determined. The cleavage rate was similar in the control and 1 B groups (control, 66.5 ± 4.92%, and 1 B, 68.74 ± 4.68%). However, the blastocyst formation rate (control, 24.9 ± 3.25%, and 1 B, 37.6 ± 2.63%; $P<0.01$) and the total cell number per blastocyst (control, 45.2 ± 1.58, and 1 B, 49.9 ± 1.56; $P<0.05$) were significantly higher in the 1 B group than in the control group. In addition, relative expression of the pluripotency marker genes POU domain, class 5, transcription factor 1 (*Pou5f1*), and caudal-type homeodomain protein CDX-2 (*CDX2*), and the antioxidant genes *SOD1* and *PRDX5*, was significantly higher in blastocysts from the 1 B group than in those from the control group ($P<0.05$).

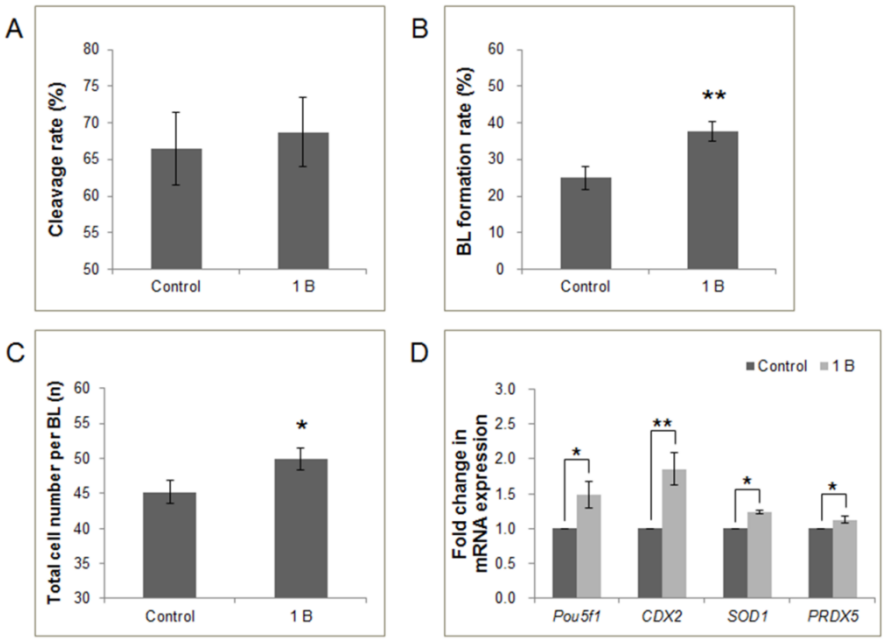

Fig. 4. Effect of β-cryptoxanthin treatment on the developmental potential and quality of embryos. Cleavage (A) and blastocyst (BL) formation (B) rates and the total cell number per blastocyst (C) in the control and 1 B groups. Relative expression levels of pluripotency marker (*Pou5f1* and *CDX2*) and antioxidant (*SOD1* and *PRDX5*) genes in blastocysts derived from oocytes matured in the presence or absence of 1 μM β-cryptoxanthin (D). GAPDH was used as an internal standard. Significant differences compared with the control group are indicated (*$p<0.05$ and **$p<0.01$). Values represent means ± standard errors of the mean of independent experiments.

4. DISSCUSTION

The present study investigated whether β-cryptoxanthin affects the IVM efficiency of porcine oocytes and/or the developmental potential and quality of embryos derived from these oocytes. Porcine oocytes were matured *in vitro* for 44 h in the presence of 0, 0.1, 1, 10, or 100 μM β-cryptoxanthin. Supplementation of IVM media with β-cryptoxanthin not only reduced oxidative stress, but also increased the rates of PB emission and blastocyst formation and the expression levels of pluripotency marker and antioxidant genes in oocytes and embryos.

β-cryptoxanthin enhanced PB emission in porcine oocytes (Table 2). PB emission can be used to assess the nuclear maturation of oocytes because it occurs during completion of the first meiotic division and progression of the second meiotic division to MII (Ogawa *et al.*, 2010; Choi *et al.*, 2013). The rate of PB emission is related to nuclear maturation. Fibroblast growth factor 10 enhances PB emission and nuclear maturation by regulating chromosome assembly and spindle alignment (Son et al., 2017). Melatonin supplementation during porcine IVM increases the rate of PB extrusion and has beneficial effects on oocyte maturation (Kang et al., 2009). Thus, β-cryptoxanthin may improve oocyte maturation by promoting progression to MII.

Gene expression is critical for oocyte maturation, and abnormalities in gene expression levels may perturb the production of competent oocytes and high quality embryos. We first checked expression of the cumulus cell expansion-related genes *SHAS2*, *HAPLN1*, and *CTSB*. Cumulus cells expand and surround an oocyte after ovulation (Downs, 1989), and proper expansion of these cells is required for normal oocyte maturation (Eppig, 2001). During cumulus cell expansion, an extracellular matrix consisting of hyaluronic acid (HA), proteoglycans, and proteins accumulates in the intercellular space and plays an important

role in oocyte maturation (Russell and Salusti, 2006). SHAS2 is an enzyme that stimulates the production and extrusion of HA (Itano et al., 1999). Expression of *SHAS2* gradually increases during COC expansion (Fülöp et al., 1997). HAPLN1 stabilizes the ECM by interacting with HA and other proteoglycans such as versican (Shi et al., 2004) and IαI (Hirashima et al., 1997). HAPLN1 supplementation during mouse oocyte IVM enhances COC expansion (Sun et al., 1999). CTSB functions as a major regulatory molecule in mammalian cell death (Angela et al., 2010) by increasing the indirect activation of caspases (Vancompernolle et al., 1998). Balboula and coworkers reported that damage of COCs by heat shock increases cumulus cell apoptosis via activating CTSB (Balboula et al., 2013). Next, we investigated expression of the maternal genes *BMP15*, *CCNB1*, and *MOS*. The paracrine factor BMP15 regulates the proliferation and expansion of cumulus cells and oocyte maturation (Hussein et al., 2006). CCNB1 and MOS are related to the cell cycle. CCNB1 binds to cyclin-dependent kinase 1 and thereby forms maturation-promoting factor (MPF), which induces the G2/M phase transition in somatic cells. In oocytes, *CCNB1* mRNA accumulates after GVBD (Zhang et al., 2011). MOS induces oncogenic transformation of somatic cells (Oskarsson et al., 1980) and thus its gene expression is tightly regulated. In addition, MOS regulates MPF activity during meiosis. MOS activates MPF during the first meiotic division and stabilizes it during the second meiotic division to arrest meiosis until sperm penetration (Sagata et al., 1989). In the present study, levels of *SHAS2* and *HAPLN1* were significantly increased and that of *CTSB* was significantly decreased in cumulus cells separated from COCs that had been matured in the presence of β-cryptoxanthin. Expression of *BMP15*, *CCNB1*, and *MOS* was increased in β-cryptoxanthin-treated oocytes. Moreover, β-cryptoxanthin treatment increased activation of p44/42 MAPK. Taken together, these results suggest that β-cryptoxanthin improves both nuclear and cytoplasmic maturation of oocytes.

The balance between the oxidative and antioxidative statuses is a major determinant of the oxidative stress level, and these statuses are mainly decided by the intracellular levels of ROS and GSH, respectively (Nasr-Esfahani et al., 1990; Boerjan and de Boer, 1990). ROS generation *in vitro* is increased by external oxygen, ultraviolet radiation, and an inefficient antioxidant system. This leads to an imbalance in the oxidative and antioxidative statuses, which can damage various molecules in oocytes and thereby reduce their quality. Many studies have sought to overcome this by optimizing the culture conditions, such as using a lower oxygen tension (Agung et al., 2010; Marques et al., 2012) or adding supplements to IVM media (Zhou et al., 2008; Kim et al., 2015). There are some evidence suggested that β-cryptoxanthin and/or its metabolites may upregulate the expression of antioxidant via the activation of the nuclear factor E2-related factor 2 (Nrf2)-dependent pathway (Kaulmann A and Bohn T, 2014). Briefly, as a transcription factor, Nrf2 is bound to the protein Kelch-like ECH-associated protein 1 (Keap1) in the cytosol. Keap1 responds to oxidative stress signals by cleaving the bond with Nrf2. Once release, Nrf2 translocates to the nucleus and binds to the antioxidant response element (ARE) that is located in the promoter of genes coding for antioxidant and/or detoxifying enzymes and scavengers. Nrf2/ARE-dependent genes code for numerous mediators of the antioxidant response, including glutamate-cysteine ligase (GCL), glutathione S-transferases (GSTs), thioredoxin, NAD(P)H quinone oxidoreductase 1 (NQO-1), and heme oxygenase 1 (HO-1) (Ben-Dor A et al., 2005).

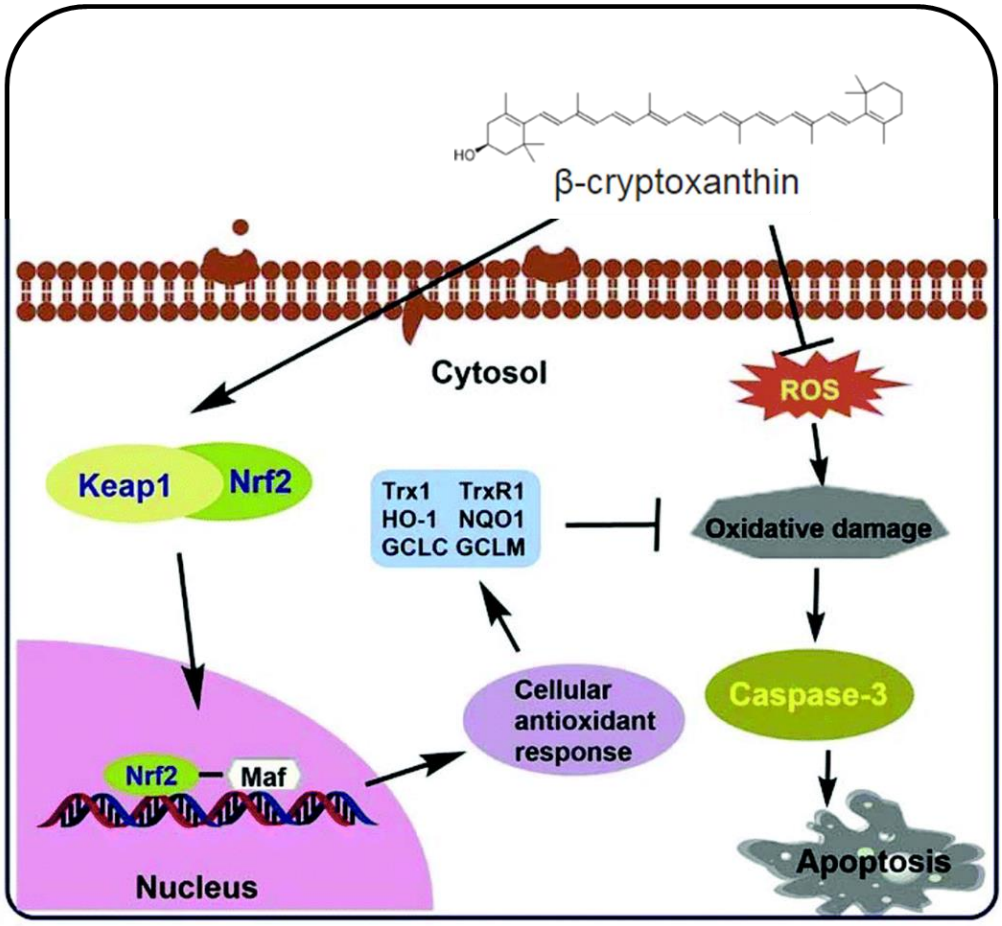

Fig. 5. Antioxidant signaling pathway of β-cryptoxanthin. β-cryptoxanthin inhibits the ROS and activates the cleaving of Nrf2-Keap1 complex. It results in activation of cellular antioxidant system.

β-cryptoxanthin has an isoprene octamer ($(C_5H_8)_8$) backbone with an ionone and an ionone derivative at end of the chain. Polyene chain formation determines the chemical reactivity of β-cryptoxanthin toward free radicals and thus its antioxidant properties. β-cryptoxanthin can be cleaved to generate retinoic acid, a form of vitamin A, in cells, and is therefore considered to be provitamin A or a vitamin A precursor (Trumbo et al., 2001; Matsumoto et al., 2007). Thus, retinoic acid activity can be used as an indicator of β-cryptoxanthin activity.

In our study, β-cryptoxanthin elicited antioxidative effects by reducing the ROS level, increasing the GSH level, and elevating the expression of antioxidant genes in oocytes (Fig. 2). We also determined the expression levels of *RARG* and *RXRA* to indirectly measure β-cryptoxanthin activity in cumulus cells and oocytes. In cumulus cells, this activity rapidly increased and then decreased during maturation, whereas it remained high in oocytes. Blastocysts derived from β-cryptoxanthin-treated oocytes highly expressed antioxidant genes (Fig. 4C). This indicates that the antioxidative effect of β-cryptoxanthin was maintained until the blastocyst stage. *SOD1* and *PRDX5* are related to the intracellular antioxidative status. SODs convert the superoxide radical, a naturally occurring and harmful ROS, into molecular oxygen and hydrogen peroxide, which are less toxic (Miao et al., 2009). Deletion of *Sod1* causes extensive oxidative damage to cells and genomic DNA in mice (Tsang et al., 2014). PRDXs detoxify ROS and thus protect cells against environmental stress (Verdoucq et al., 1999; Wood et al., 2003). PRDX5 is targeted to mitochondria and peroxisomes (Verdoucq et al. 1999; Zhou et al. 2000) and is characterized as a thioredoxin peroxidase. Collectively, these results suggest that β-cryptoxanthin is a powerful antioxidant.

The *in vitro* development and quality of embryos were enhanced in the β-cryptoxanthin-treated group. Expression of *Pou5f1* and *CDX2* was increased in blastocysts derived from oocytes matured in the presence of β-cryptoxanthin (Fig. 4). *Pou5f1* is related to pluripotency and is highly expressed in primordial germ cells, the inner cell mass, and

embryonic stem cells (Pesce and Scholer, 2001 and Kehler et al., 2004). Moreover, Pou5f1 is a key regulator of the molecular events that establish the developmental competence of mouse oocytes (Zuccotti et al., 2008). CDX2 is related to the formation and maintenance of trophectoderm (TE) (Yamanaka et al., 2006). In mouse embryos lacking CDX2, although a TE-like structure forms, outgrowth and implantation of TE into the uterus fail (Strumpf et al., 2005). This indicates that addition of β-cryptoxanthin to IVM media enhances the developmental potential and quality of porcine embryos and the maturation efficiency of porcine oocytes.

In conclusion, our data demonstrate that supplementation of IVM media with β-cryptoxanthin significantly improves the maturation of porcine oocytes by regulating oxidative stress, nuclear and cytoplasmic maturation, and relative gene expression in cumulus cells and oocytes. Moreover, β-cryptoxanthin treatment enhances the development and quality of embryos derived from these oocytes. Therefore, the antioxidant β-cryptoxanthin can prevent oxidative stress in oocytes and embryos cultured *in vitro* during the production of cloned animals and ART.

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ABSTRACT IN KOREAN

산화 스트레스는 체외에서 성숙된 난자의 품질 감소의 대표적인 원인이 되는 요인이다. 본 연구에서는 돼지 난자의 체외 성숙 과정과 이들 난자로부터 만들어진 체외생산 배아의 발달에 항산화제의 일종인 베타-크립토잔틴이 미치는 영향을 조사하였다. 돼지의 난소로부터 회수된 미성숙 난자는 각각 0, 0.1, 1, 10, 100 μM 의 베타-크립토잔틴을 포함하는 체외성숙용 배양액에서 체외성숙이 유도되었다 (대조군, 0.1 B, 1 B, 10 B 와 100 B). 본 실험의 결과, 44 시간 체외성숙 후 제 1 극체 방출률은 1 B 처리군에서 대조군에 비해 증가하였고, 1 B 처리군에서는 성숙과 관련된 유전자의 발현이 난구세포와 난자 모두에서 유의적으로 향상되었다. 또한 난자의 성숙이 진행되는 전 기간 동안 1 B 처리군 내 난자의 세포질에서 활성산소종의 수준이 현저하게 낮아지고 글루타티온의 수준이 유의적으로 증가하였다. 이와 유사하게 항산화 유전자인 과산화물제거효소 1 (SOD1)과 페록시레독신 5 (PRDX 5)의 발현도 유의하게 향상되었다. 성숙이 완료된 난자에 단위발생을 유도한 후, 난할률은 대조군과 1 B 처리군에서 차이가 없었지만, 배반포 형성률은 1 B 처리군이 유의적으로 높았다. 배반포 당 총 세포수와 배아 발달과 관련된 유전자 및 항산화 유전자의 발현 수준 모두 대조군에 비해 1 B 처리군에서 유의하게 높았다. 이와 같은 결과는 난자의 체외성숙 동안 베타-크립토잔틴의 처리는 난자 내의 산화스트레스를 감소시킴으로써 난자 자체 및 이로부터 생성된 배아의 발달 잠재력과 질적 향상을 유도할 수 있음을 보여준다.

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