Master's Thesis

Comparison of Three Antioxidants in Chemical and Biological Assays on Porcine Oocytes during Aging *in vitro*

Chan-Oh Park

Department of Biotechnology

GRADUATE SCHOOL

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심사위원장 류 기 중 ㊞

위 원 김 은 영 ㊞

위 원 박 세 필 ㊞

제주대학교 대학원

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Chan-Oh Park

(Supervised by Professor Se-Pill Park)

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This thesis has been examined and approved.

Chairperson of the supervising committee

Professor Key-Zung Riu, Ph.D., **College of Applied Life Sciences**,**Jeju National University**

Professor Eun-Young Kim, **Ph.D.**, **College of Applied Life Sciences**,**Jeju National University**

Professor Se-Pill Park, **Ph.D., College of Applied Life Sciences**,**Jeju National University**

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Abstract

In this study, we investigated which of the β-cryptoxanthin (BCX), hesperetin (HES), or icariin (ICA) antioxidants were more effective for *in vitro* maturation (IVM) of porcine oocytes. The antioxidant properties were assessed with aged porcine oocytes and embryos by comparing DPPH, reducing power, and H_2O_2 scavenging activity assays. The chemical assay results demonstrated that BCX had a greater DPPH scavenging activity and reducing power than HES and ICA, compared with controls. However, the H_2O_2 scavenging activity of the antioxidants was similar when tested at the optimal concentrations of 1 μM BCX (BCX-1), 100 μM HES (HES-100), and 5 μM ICA (ICA-5). The biological assay results showed that BCX-1 treatment was more effective in inducing a significant reduction in reactive oxygen species (ROS), improving glutathione levels, and increasing the expression of antioxidant genes. In addition, BCX-1 inhibited apoptosis by increasing the expression of anti-apoptotic genes and decreasing pro-apoptotic genes in porcine parthenogenetic blastocysts. BCX-1 also significantly increased the blastocyst formation rate compared with the aging control group, HES-100 and ICA-5. This study demonstrates that damage from ROS produced during oocyte aging can be prevented by supplementing antioxidants into the *in vitro* maturation medium, and BCX may be a potential candidate to improve assisted reproductive technologies.

Keywords: *in vitro* aging, porcine oocyte, ß-cryptoxanthin, hesperetin, icariin.

1. Introduction

To achieve rapid growth and differentiation, an embryo must obtain sufficient energy by utilizing products of the mitochondrial electron transport chain, such as ATP, NADPH, and oxygen. Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radical, are constant by-products of this process that are quickly eliminated by antioxidant defenses (Halliwell, 1991). Endogenous enzymatic antioxidants, including superoxide dismutase, catalase, and glutathione peroxidase, or non-enzymatic compounds, such as bilirubin and albumin, regulate excess ROS (Francenia Santos-Sánchez, 2019). Although these defense systems reduce the amount of ROS, this can be counteracted by an imbalance of oxidizing and reducing compounds induced by oocyte aging (Moldogazieva, Mokhosoev, Feldman, & Lutsenko, 2018; Wang, Branicky, Noe, & Hekimi, 2018). Oocyte aging is a process normally occurring while the unfertilized oocytes pass their reproductive age and mature inside the female ovary (Nemecek, Dvorakova, Heroutova, Chmelikova, & Sedmikova, 2017). As a result of oocyte aging, there is an imbalance of oxidation and reduction that results in the excess generation of ROS, which leads to developmental arrest, DNA damage, apoptosis, and lipid peroxidation (Takahashi, 2012).

To prevent ROS-induced oxidative stress upon aging oocytes and to maintain quality for assisted reproduction technologies, several researchers have tested various antioxidants such as ubiquinol-10, kaempferol, melatonin, β-cryptoxanthin, hesperetin and icariin (W. J. Kim et al., 2019; Mehaisen et al., 2015; Niu et al., 2020; Park et al., 2018; Yao et al., 2019; Yoon et al., 2020). β-cryptoxanthin (BCX) is a carotenoid abundant in fruits such as tangerines and peaches, and is a known precursor of vitamin A. This vitamin has several useful functions including antioxidant capacity and cell-to-cell communication (Burri, La Frano, & Zhu, 2016). In addition, BCX prevents the development of various cancers (e.g.,

lung, liver, prostate, breast, colorectal, and stomach) (Lim & Wang, 2020). Hesperetin (HES) is a flavanone class of flavonoid found in citrus fruits such as oranges and grapefruit (H. K. Kim, Jeong, Lee, Park, & Choi, 2003). This compound has several beneficial effects such as antioxidant and anti-inflammatory capacity (Muhammad, Ikram, Ullah, Rehman, & Kim, 2019). Icariin (ICA) is a chemical compound classified as a prenylated flavonol glycoside, a type of flavonoid. It is the 8-prenyl derivative of kaempferol 3,7-O-diglucoside. ICA can be found and isolated from several plants belonging to the genus Epimedium, which is commonly known as Horny Goat Weed (J. J. Liu, Li, & Wang, 2006). Flavonoid glycosides, which can be widely found in nature, are proven to have many pharmacological activities including antioxidant, anti-inflammatory, and anti-tumor effects (J. Liu et al., 2019).

In this study, we wanted to determine which antioxidant is more effective against oxidative stress induced by oocyte aging by comparing chemical and biological analyses. To compare the chemical antioxidant capacity of the three antioxidants (BCH, HES, and ICA), DPPH, reducing power, and H_2O_2 scavenging activity assays were conducted. To compare the biological antioxidant capacity of the antioxidants, oocytes were experimentally aged by 24 h of incubation in *in vitro* maturation medium following 44 h of *in vitro* maturation. A previous study showed that 24 h of rapamycin treatment supplemented to *in vitro* maturation medium rescued the poor developmental capacity of aged porcine oocytes after an initial 44 h of IVM treatment (Lee et al., 2014). The levels of ROS and glutathione (GSH) were analyzed by measuring the expression of antioxidant genes (*SOD1*, *SOD2*, and *NFE2L2*), anti-apoptotic genes (*BCL2L1* and *BIRC5*), pro-apoptotic genes (*CASP3* and *FAS*), and developmental genes (*POU5F1, NANOG*, and *SOX2*), as well as the apoptosis rate in aged porcine oocytes and embryos treated with or without antioxidants. The developmental integrity and quality of the embryos were compared from oocytes treated in the presence or absence of antioxidants. These results suggest that BCX-1 is a more effective antioxidant

than HES-100 or ICA-5 during *in vitro* maturation of porcine oocytes and embryos. These findings may be applicable to *in vitro* fertilization to help prevent aging of oocytes *in vitro*.

2. Materials & Methods

2.1. Chemicals and reagents

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

2.2. DPPH free radical scavenging assay

The DPPH (2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) assay, originally reported by Blois (Blois, 1958), was used to measure the free radical scavenging activity of the antioxidants with some modification. Stock solutions of BCX (Extrasynthese, Lyon, France), HES, and ICA were diluted in DMSO and serial dilutions were prepared (0.04, 0.08, 0.12, 0.16, and 0.20 μg/mL BCX; 1, 2, 3, 4, and 5 mg/mL HES; 10, 20, 30, 40, and 50 mg/mL ICA). Briefly, 20 μL of the samples were diluted in DMSO and added to 180 μL of a 167 μM DPPH working solution. The absorbance of the solution was measured at 517 nm with a SpectraMAX i3 (Molecular Devices, LLC; Sunnyvale, CA). The DPPH free radical scavenging activity was calculated using the following equation (1):

$$\text{DPPH scavenging effect (\%)} = \frac{\text{Ac}-\text{At}}{\text{Ac}} \times 100 \quad (1)$$

, where Ac is the absorbance value of the control reaction (blank sample treated with no antioxidant), and At is the absorbance in the presence of the antioxidants.

2.3. Reducing power assay

The reducing power of the antioxidants at different concentrations (125, 250, 500, and 1000 μM) was determined using a method described by Gülçin (Gülçın, Oktay, Kıreççı, & Küfrevıoğlu, 2003). Briefly, 0.1 mL sample solution was combined with 0.25 mL phosphate

buffer (0.2 M, pH 6.6) and 0.25 mL potassium ferricyanide [$K_3[Fe(CN)_6]$] (1%; Poly Scientific R&D, Bay Shore, NY), mixed, and then incubated at 50°C for 20 min. Trichloroacetic acid (10%, 0.25 mL) was added to the samples and centrifuged for 10 min at 1700 × *g* (25°C). After centrifugation, 0.25 mL from the upper layer of the solution was transferred to a new tube, and mixed with 0.25 mL distilled water and 0.05 mL ferric chloride [$FeCl_3$] (0.1%). The absorbance of the reaction was measured at 700 nm with a SpectraMAX i3 (Molecular Devices, LLC). An increased absorbance value indicated an increased reducing power.

2.4. H_2O_2 scavenging activity assay

The H_2O_2 scavenging activity of the antioxidants was determined by the method described by Ruch RJ (Ruch, Cheng, & Klaunig, 1989) with slight modifications. Briefly, a 4 mM solution of H_2O_2 was prepared in phosphate-buffered saline (PBS, pH 7.4) at 20°C. H_2O_2 concentration was determined spectrophotometrically at an absorbance of 230 nm. The three antioxidants were each dissolved in DMSO and then added to the H_2O_2 solution at final concentrations of 1 μM BCX, 100 μM HES, and 5 μM ICA at 20°C. A control reaction was prepared containing only 0.1% of DMSO. Ten minutes after incubation, the amount of H_2O_2 in each sample was determined using a SpectraMAX i3 (Molecular Devices). The H_2O_2 scavenging activity was calculated using the following equation (2):

$$H_2O_2 \text{ scavenging effect } (\%) = \frac{Ac - At}{Ac} \times 100 \quad (2)$$

, where Ac is the absorbance value of the control reaction (blank sample treated with no antioxidant), and At is the absorbance in the presence of the antioxidants.

2.5. Oocyte collection and IVM

Prepubertal porcine ovaries were collected from a local slaughterhouse within 2 h of

slaughter and transported to the laboratory in saline supplemented with 75 μg/mL penicillin G and 50 μg/mL streptomycin sulfate at 30–33°C. Cumulus-oocyte complexes (COCs), 2–8 mm in diameter, were aspirated from the ovarian follicle using an 18-gauge needle attached to a disposable 10 mL syringe. COCs were washed thrice 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) 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. The COCs were cultured in petri dishes under a layer of mineral oil and incubated for 44 h at 38.8°C with 5% CO_2.

2.6. Antioxidant treatment during aging of matured porcine oocytes

Oocytes were matured in a 4-well tissue culture dish containing 500 μL TCM-199, covered with a layer of mineral oil, by incubation for 68 h (with the exception of a control group for 44 h) at 38.8°C with 5% CO_2. After maturation (44 h), metaphase-II (MII)-stage oocytes were transferred into dishes containing TCM-199 supplemented with 1% DMSO, 1 μM BCX, 100 μM HES, and 5 μM ICA, which is refer to aging, BCX-1, HES-100 and ICA-5, and incubated for 24 h. After antioxidant treatment, the oocytes were collected and maturity was assessed.

2.7. Parthenogenesis and embryo culture

Following oocyte maturation, cumulus cells were isolated by repeated manual pipetting in the presence of 1 mg/mL hyaluronidase for 2–3 min. Parthenogenesis (PA) was induced by treating oocytes with porcine zygote medium (PZM)-5 containing 0.4% (w/v) BSA (IVC medium) and 5 μM Ca^{2+} ionomycin (Sigma) for 5 min. Following 3 h of incubation in IVC

medium containing 7.5 μg/mL cytochalasin B (Sigma), embryos were washed thrice with IVC medium before being cultured for 7 days at 38.8°C with 5% CO_2. Oocytes and embryos were washed in Dulbecco's PBS (DPBS) and either fixed in 3.7% (w/v) paraformaldehyde for 20 min and stored at 4°C, or snap-frozen in liquid nitrogen and stored at -70°C, depending on the experiment.

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

The 2′,7′-Dichlorofluorescin diacetate (DCFHDA) and 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CMF_2HC) were used to determine the intracellular levels of ROS and GSH, respectively, as previously described by Yang HW (Yang et al., 1998) and You J (You, Kim, Lim, & Lee, 2010) with slight modifications. Briefly, cumulus cells were isolated from the COCs by repeated manual pipetting in the presence of 0.1% (w/v) hyaluronidase. Denuded oocytes were incubated in DPBS containing 50 μM DCFHDA or 100 μM CMF_2HC in the dark for 20 min at 38.8°C with 5% CO_2. After, oocytes were washed a minimum of five times with DPBS containing 0.1% (w/v) BSA to completely remove excess dye and then were immediately analyzed by epifluorescence microscopy (Olympus; Shinjuku, Tokyo). The ROS level was measured using excitation and emission wavelength ranges of 450–490 and 515–565 nm, respectively. The excitation and emission wavelengths of CMF_2HC were 371 and 464 nm, respectively. Grayscale images were acquired with a digital camera (Shinjuku, Tokyo) attached to the microscope, and mean grayscale values were calculated using ImageJ software (NIH, Bethesda, MD). Background fluorescence values were subtracted from the final values before statistical analysis. A total of 6–7 independent experiments were performed with 10–20 oocytes per experiment.

2.9. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and

Hoechst staining

At 7 days after PA, blastocysts were fixed, washed a minimum of three times with PBS containing 0.1% BSA, and then incubated with 0.1% Triton X-100 at 38.8°C with 5% CO_2 for 30 min. Blastocysts were incubated with fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase (In Situ Cell Death Detection Kit; Roche, Mannheim) in the dark for 1 h at 38.8°C. The numbers of mitotic and apoptotic cells were recorded. Nuclei were stained with Hoechst 33342 (1 μg/mL) for 30 min, and 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. Six independent experiments were performed.

2.10. mRNA extraction and complementary DNA (cDNA) synthesis

For PCR sample preparation, mRNA was isolated from a minimum of three biological replicates, with 20 oocytes per replicate, using a Dynabeads mRNA Direct Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The mRNA was suspended in 10 μL elution buffer that was 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.

2.11. Real-time RT-PCR

Real-time RT-PCR was performed as previously described by Lee SE (Lee, Sun, Choi, Uhm, & Kim, 2012) using the primer sets listed in Table **1.** Samples were loaded in a final reaction volume of 20 μL containing SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Analysis was performed using a StepOnePlus Real-time PCR System (Applied Biosystems) with the following program settings: 10 min at 95°C, followed by 39

cycles of 15 s at 95°C, and 60 s at 54 or 60°C. Samples were then cooled to 12°C. Relative gene expression levels were analyzed by the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001) after normalization against the housekeeping gene *ACTB*. Three independent experiments were performed.

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

Gene	GenBank accession no.	Primer sequence	Annealing temperature (°C)	Size (bp)
ACTB	AY550069.1	F: AGATCATGTTCGAGACCTTC R: GTCAGGATCTTCATGGGTAGT	54	220
SOD1	GU944822.1	F: GTGTTAGTAACGGGAACCAT R: GGATTCAGGATTGAAGTGAG	54	120
SOD2	NM_214127.2	F: AGACCTGATTACCTGAAAGC R: CTTGATGTACTCGGTGTGAG	54	110
NFE2L2	Gu991000.1	F: CTATGGAGACACACTGCTTG R: ACAGGCTGTGTTTTAGGACT	54	99
POU5F1	NM_001113060	F: AGTGAGAGGCAACCTGGAGA R: TCGTTGCGAATAGTCACTGC	60	166
NANOG	F_DQ447201	F: TTCCTTCCTCCATGGATCTG R: ATCTGCTGGAGGCTGAGGTA	60	214
SOX2	F_EU503117	F: GCCCTGCAGTACAACTCCAT R: GCTGATCATGTCCCGTAGGT	60	216
BCL2L1	NM_214285.1	F: GGTTGACTTTCTCTCCTACAAG R: CTCAGTTCTGTTCTCTTCCAC	54	118
BIRC5	NM_214141.1	F: CTTCTGCTTCAAAGAGCTG R: GGCTCTTTCTTTGTCCAGT	54	154
FAS	AJ001202.1	F: GAGAGACAGAGGAAGACGAG R: CTGTTCAGCTGTATCTTTGG	54	194
CASP3	NM_214131	F: GACTGCTGTAGAACTCTAACTGG R: ATGTCATCTTCAGTCCCACT	54	110

F, forward; R, reverse.

2.12. Statistical analysis

All experimental data were anin vitoroalyzed using the general linear model procedure using the Statistical Analysis System software (SAS User's Guide 1985, Statistical Analysis System Inc., Cary, NC). Tukey's multiple range test was used to determine significant differences. *P*-values were considered as significant ($^{*}P < 0.05$, $^{**}P < 0.01$).

3. Results

3.1. Improved DPPH scavenging activity of BCX compared with HES and ICA

The scavenging ability of BCX, HES, and ICA to reduce DPPH free radicals was determined by measuring the decrease in absorbance. The result showed that scavenging ability was dependent on antioxidant concentration (Table 2). To compare the scavenging efficiency, an estimate for the concentration of antioxidants required to inhibit 50% of DPPH radicals (IC50) was extrapolated from a linear regression analysis (Fig. 1). The resulting IC50 values of BCX, HES, and ICA were determined to differ significantly ($P < 0.001$) at 0.109 mg/mL BCX, 3.255 mg/mL HES, and 20.517 mg/mL ICA, respectively (Table 2).

Table 2. DPPH scavenging activity of the three antioxidants tested at different concentrations.

Antioxidant	Concentration (mg/mL)	Absorbance at 517 nm	DPPH scavenging activity (%)	IC 50 (mg/mL)
BCX	0.04	0.5792 ± 0.0010	22.07 ± 0.09[a]	0.1089 ± 0.0007[a]
	0.08	0.4456 ± 0.0017	40.04 ± 0.20[d]	
	0.12	0.3269 ± 0.0032	56.02 ± 0.40[g]	
	0.16	0.1937 ± 0.0039	73.94 ± 0.51[j]	
	0.20	0.1108 ± 0.0038	85.10 ± 0.50[l]	
HES	1	0.5837 ± 0.0065	22.83 ± 0.80[a]	3.2550 ± 0.0734[b]
	2	0.4708 ± 0.0073	37.75 ± 0.91[c]	
	3	0.3975 ± 0.0080	47.45 ± 1.01[e]	
	4	0.3316 ± 0.0075	56.16 ± 0.96[g]	
	5	0.2891 ± 0.0069	61.78 ± 0.88[h]	
ICA	10	0.5208 ± 0.0021	31.54 ± 0.27[b]	20.5167 ± 0.0126[c]
	20	0.3649 ± 0.0025	52.04 ± 0.32[f]	
	30	0.2485 ± 0.0025	67.33 ± 0.32[i]	
	40	0.1902 ± 0.0020	75.00 ± 0.26[j]	
	50	0.1472 ± 0.0033	80.65 ± 0.43[k]	

[a-l]$P < 0.05$, [a-c]$P < 0.001$.

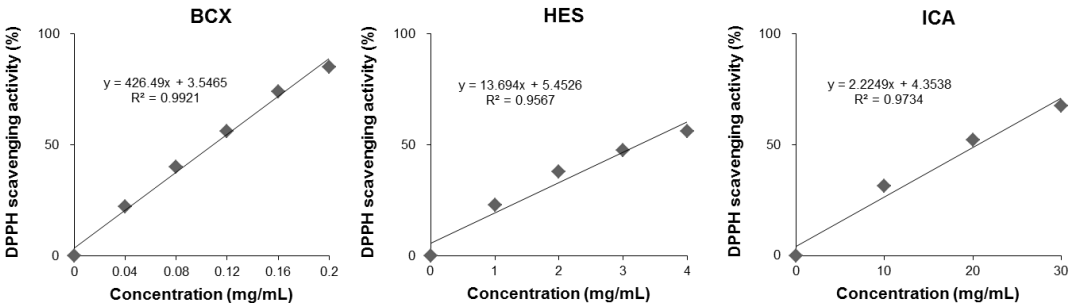

Figure 1. DPPH scavenging activity of the antioxidants at different concentrations. The results are represented by concentration vs. DPPH scavenging activity (%). Data are derived from three independent experiments represented by the mean ± SEM.

3.2. Improved reducing power of BCX compared with HES and ICA

The reducing power of the antioxidants was correlated with concentration in the range of 125 to 1000 μM. When measured at an absorbance of 700 nm, the antioxidant reducing powers were statistically different ($P < 0.05$, Fig. 2A). BCX had a greater reducing power than both HES and ICA at the same concentration. Compared with controls, there were significant differences in the reducing power relative to concentration; however, BCX was the most effective even at very low concentrations (Fig. 2A). Additionally, the reducing power at the lowest concentration of HES was significantly stronger than the highest concentration of ICA ($P < 0.05$, Fig. 2A).

3.3. BCX, HES, and ICA demonstrate similar H_2O_2 scavenging activities

To compare the H_2O_2 scavenging activity, a H_2O_2 scavenging assay was performed using the optimal concentrations of each antioxidant in IVM porcine oocytes**.** The sample groups BCX-1, HES-100, and ICA-5 scavenged approximately 5% of H_2O_2 compared with the control, although this effect was not significantly different (Fig. 2B). Although the optimal concentration of BCX was lower than HES and ICA, BCX had an equivalent H_2O_2 scavenging activity compared with HES-100 or ICA-5 (Fig. 2B).

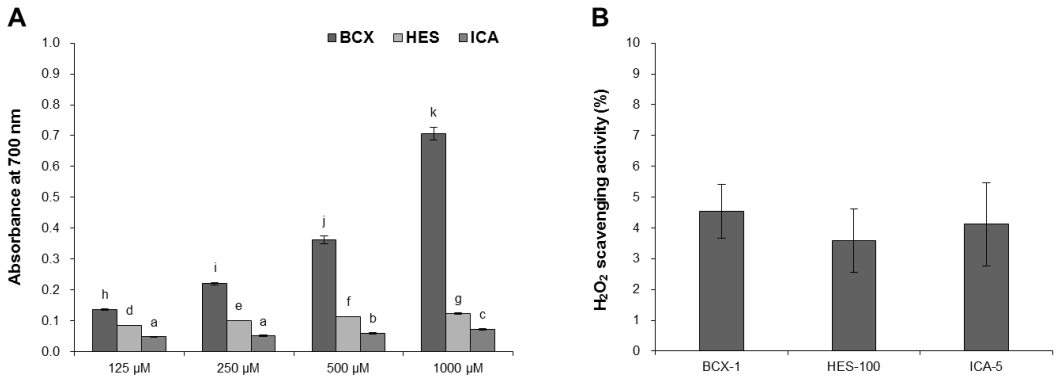

Figure 2. The reducing power and H_2O_2 scavenging activity of BCX, HES, and ICA. (a) The reducing power of the antioxidants tested at different concentrations and measured at an absorbance of 700 nm ($^{a\text{-}k}P < 0.05$). (b) The H_2O_2 scavenging activity of the antioxidants tested at optimal concentration during *in vitro* maturation of porcine oocytes. Data are derived from three independent experiments represented by the mean ± SEM.

3.4. BCX reduces oxidative stress by increasing GSH in matured porcine oocytes

To assess oxidative stress in matured porcine oocytes, ROS and GSH levels were measured on porcine oocytes initially matured for 44 h (control) or for an additional 24 h (total of 68 h) in the presence of 1% DMSO, 1 μM BCX, 100 μM HES, and 5 μM ICA, which were then referred to as the aging, BCX-1, HES-100, and ICA-5 groups, respectively (Fig. 3A). The GSH levels were significantly increased in the BCX-1, HES-100, and ICA-5 sample groups compared with the control ($P < 0.01$, Fig. 3B). Despite slightly lower GSH levels in the HES-100 (103.42 ± 4.65 pixels/oocyte) and ICA-5 (107.28 ± 3.67 pixels/oocyte) groups compared with the control group, the BCX-1 (115.97 ± 6.80 pixels/oocyte) result was similar to the control group (111.91 ± 3.19 pixels/oocyte) but not to the aging group (42.47 ± 3.12 pixels/oocyte) (Fig. 3B). The staining intensity of ROS was significantly lower ($P < 0.01$) in the control (113.73 ± 2.49 pixels/oocyte) and BCX-1 (104.13 ± 2.52 pixels/oocyte) groups compared with the aging (124.18 ± 1.77 pixels/oocyte) group, however, there was no difference between aging, HES-100 (123.19 ± 3.87 pixels/oocyte), and ICA-5 (123.57 ± 3.07 pixels/oocyte) (Fig. 3C). Real-time RT-PCR was performed to investigate the effect of antioxidants on mRNA expression of nuclear factor erythroid 2-like 2 (*NFE2L2)*, superoxide dismutase 1 (*SOD1*), and superoxide dismutase 2 (*SOD2*) in porcine oocytes at MII (Fig. 3D, 3E, 3F). The BCX-1 group significantly increased the expression of *SOD1* and *SOD2* compared with the aging and ICA-5 sample groups ($P <0.01$, Fig. 3D, 3F). The HES-100 group increased the expression of *SOD1* but not *SOD2,* compared with the aging and ICA-5 sample groups ($P < 0.05$, Fig. 3D). The expression level of *NFE2L2* was not different between any group (Fig. 3E).

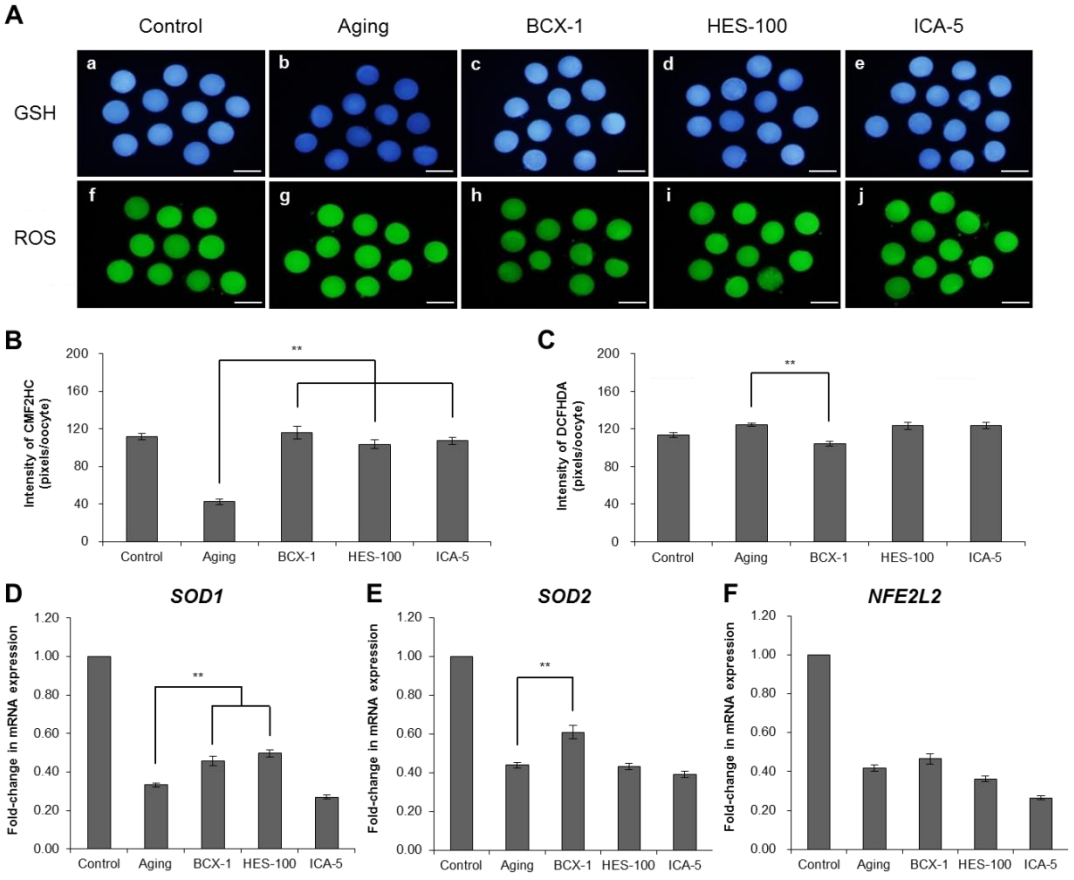

Figure 3. Antioxidant activity against oxidative stress during aging of porcine oocytes. (a) Images of oocytes stained with CMF_2HC (blue), DCFHDA (green) for assessing GSH (a–e) and ROS (f–j)). Quantification of the fluorescence intensities of (b) CMF_2HC and (c) DCFHDA ($^{**}P < 0.01$). Relative expression of antioxidant genes (d) *SOD1*, (e) *SOD2*, and (f) *NFE2L2* in MII-stage porcine oocytes ($^{**}P < 0.01$). Data are derived from three independent experiments represented by the mean ± SEM. Scale bars = 120 μM.

3.5. BCX enhanced developmental competence in aging oocytes

Comparing the development effect of the antioxidants, the percentages of oocytes that survived to MII-stage were not significantly different between control (83.77% ± 1.58%) and treatment groups (BCX-1, 81.79% ± 4.07%; HES-100, 74.70% ± 5.78%; ICA-5, 72.77% ± 5.30%), except the aging group (72.32% ± 4.34%; Fig. 4A). Oocyte survival was highest in and most similar between the BCX and control groups. Following PA, the percentage of oocytes that developed into the 2–4-cell cleavage stage was similar between groups (control, 63.26% ± 5.47%; aging, 75.97% ± 5.73%; BCX-1, 67.64% ± 4.22%; HES-100, 67.57% ± 2.80%; ICA-5, 72.18% ± 5.21%; Fig. 4A). The percentage of cleavage embryos that developed into blastocysts by day 7 was significantly higher ($P < 0.01$) in the BCX-1 (32.87% ± 3.72%) and control (35.89% ± 2.81%) group than in the aging (18.46% ± 1.86%), HES-100 (23.04% ± 4.47%), and ICA-5 (24.75% ± 1.73%) groups (Fig. 4A). Additionally, the expression of important developmental genes, including POU domain, class 5, transcription factor 1 (*POU5F1*), North American Network Operators' Group Homeobox protein (*NANOG*), and SRY (sex determining region Y)-box **2** (*SOX2*), was investigated in porcine blastocysts that had been cultured for 7 days (Fig. 4B, 4C, 4D). BCX-1 treatment significantly increased the expression of *POU5F1* ($P < 0.05$) and *SOX2* ($P < 0.01$) compared with the aging, HES-100, and ICA-5 groups (Fig. 4B, 4D). Treatment with HES-100 and ICA-5 increased the expression of *NANOG* ($P < 0.05$) but not *POU5F1* or *SOX2,* compared with the aging or BCX-1 groups (Fig. 4C).

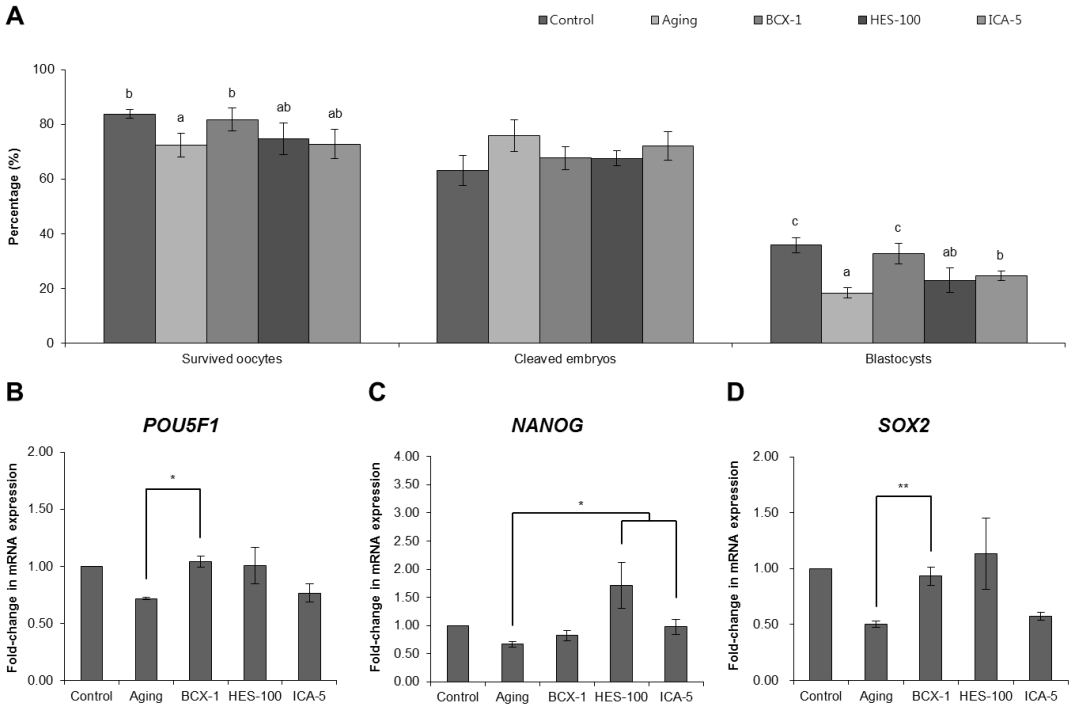

Figure 4. Effect of antioxidant treatment during aging of porcine oocytes on subsequent embryo development and expression of developmental genes in porcine blastocysts. (a) The percentage of survived oocytes ($^{a\text{-}b}P < 0.05$), cleaved embryos, and formed blastocysts ($^{a\text{-}c}P < 0.05$) during *in vitro* maturation and subsequent culture for 7 days. Relative expression of the developmental genes (b) *POU5F1*, (C) *NANOG*, and (D) *SOX2* in porcine blastocysts ($^{*}P < 0.05$, $^{**}P < 0.01$). Data are derived from three independent experiments represented by the mean ± SEM.

3.6. BCX improves blastocyst development compared with HES and ICA

To compare whether BCX, HES, or ICA treatment during IVM of porcine oocytes influenced the subsequent embryonic development and integrity, the blastocyst formation rate was assessed at 7 days post-PA (Fig. 4A). The total number of cells per blastocyst was significantly higher ($P < 0.01$) in the control (71.5 ± 12.2) and BCX-1 (69.7 ± 8.8) groups compared with the aging, HES-100, and ICA-5 groups (aging, 52.4 ± 7.6; HES-100, 59.4 ± 4.0; ICA-5, 57.8 ± 4.1) (Fig. 5A). Genomic DNA fragmentation was assessed by a TUNEL assay to detect the percentage of apoptotic cells in the blastocysts. The percentage of apoptotic cells was significantly lower ($P < 0.01$) in the control (1.3% ± 0.7%), BCX-1 (2.4% ± 0.7%), and HES-100 (2.6% ± 0.8%) groups compared with the aging and ICA-5 group (control, 1.3% ± 0.7%; ICA-5, 5.4% ± 1.1%) (Fig. 5B). Additionally, the expression of anti-apoptotic genes including BCL2-like 1 (*BCL2L1*) and baculoviral IAP repeat-containing 5 (*BIRC5*), and pro-apoptotic genes such as caspase-3 (*CASP3*) and Fas cell-surface death receptor (*FAS*), were investigated in porcine blastocysts (Fig. 5C–F). Treatment with BCX-1 significantly increased the expression of *BCL2L1* ($P < 0.01$, Fig. 5C) and *BIRC5* ($P < 0.05$, Fig. 5D), and decreased the expression of *CASP3* ($P < 0.01$, Fig. 5E) but not *FAS*. Treatment with HES-100 significantly increased the expression of *BCL2L1* ($P < 0.01$, Fig. 5C) and decreased the expression of both *CASP3* ($P < 0.05$, Fig. 5E) and *FAS* genes ($P < 0.01$, Fig. 5F). Treatment with ICA-5 significantly increased the expression of *BCL2L1* ($P < 0.05$, Fig. 5C) and *BIRC5* ($P < 0.05$, Fig. 5D), and decreased the expression of *CASP3* ($P < 0.01$, Fig. 5E) and *FAS* genes ($P < 0.01$, Fig. 5F).

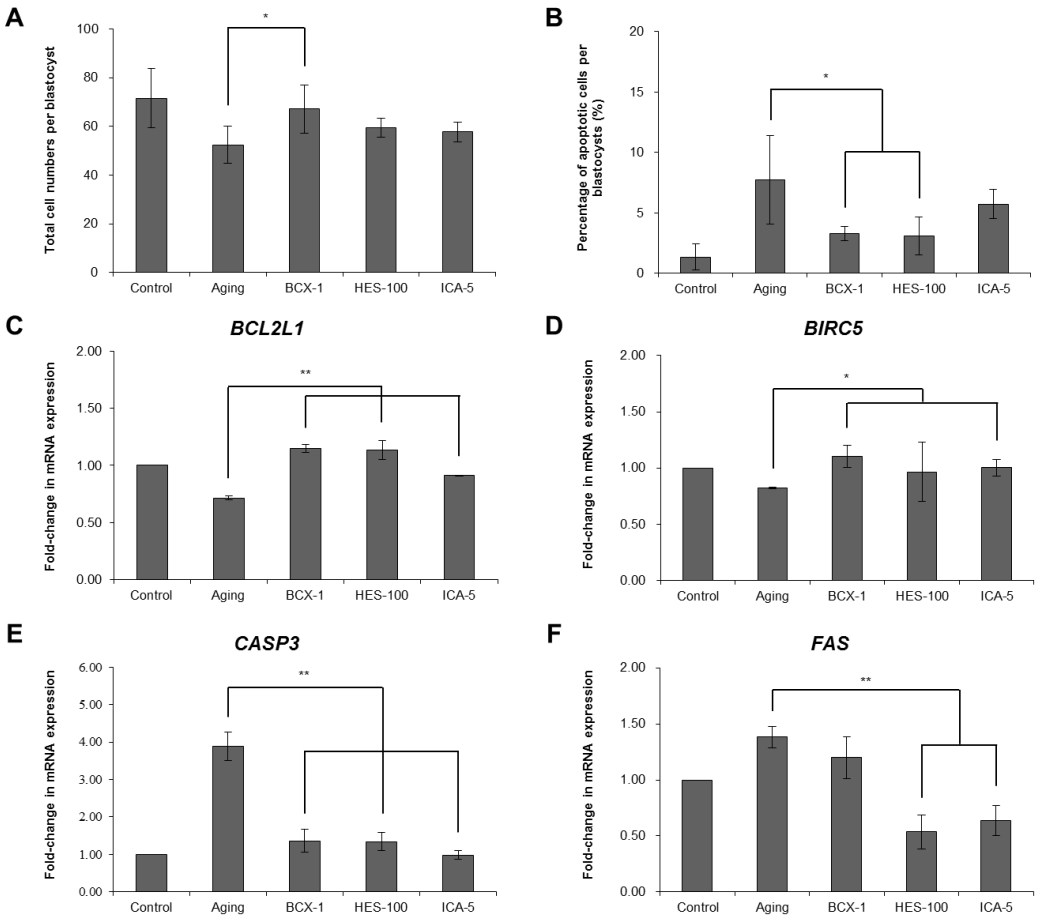

Figure 5. Effect of the antioxidant treatment during aging of porcine blastocysts on cell survival and apoptosis. (a) Total cell number and (b) percentage of apoptotic cells in porcine blastocysts ($^{*}P < 0.05$). Expression of anti-apoptotic genes (c) *BCL2L1* and (d) *BIRC5*, and pro-apoptotic genes (e) *CASP3* and (f) *FAS,* in porcine blastocysts ($^{*}P < 0.05$, $^{**}P < 0.01$). Data are derived from three independent experiments represented by the mean ± SEM.

Discussion

This study investigated whether the antioxidants BCX, HES, and ICA could effectively reduce oxidative stress induced by oocyte aging. Although the mechanism by which oocytes are preserved from the normal aging process *in vitro* is unknown, we tried to measure antioxidant activity by chemical and biological assays. First, we measured the chemical antioxidant capacity of BCX, HES, and ICA with the DPPH assay. DPPH is a stable free radical that can be used to measure the radical scavenging activity of antioxidants. The DPPH assay is a short and simple method to measure the electron-donating ability of antioxidants during lipid oxidation (Kikuzaki, Hisamoto, Hirose, Akiyama, & Taniguchi, 2002). To compare chemical antioxidant activity, we calculated the IC 50 value representing the concentration of the sample required to inhibit 50% of the DPPH radical. To calculate an approximate IC 50 value with a 95% confidence level, a linear regression line was generated against antioxidant concentration, based on the guidelines by Sebaugh JL (Sebaugh, 2011). According to the linear regression formula (Fig. 1), the IC 50 values of BCX, HES, and ICA were 0.1089 mg/mL, 3.2550 mg/mL, and 20.5167 mg/mL, respectively, as shown in Table 2. As the IC 50 value directly represents the sample concentration required to inhibit 50% of DPPH, we assume that the antioxidant activity of BCX is approximately 30-fold stronger than that of HES, and approximately 200-fold stronger than that of ICA, at any given concentration (mg/mL). Most papers are not considering final sample concentration by Sample and DPPH reaction to calculate IC 50 value (Chen & Huang, 2019; Hara et al., 2018; Lopez-Martinez, Santacruz-Ortega, Navarro, Sotelo-Mundo, & Gonzalez-Aguilar, 2015). As the final sample concentration tested in the DPPH assay was not included in the linear regression analysis, so the values should be diluted 10-fold (data not shown) to apply for

biological experiments or supplement to bioreagents. In this study, three antioxidants were supplemented with *in vitro* maturation medium; modified IC 50 was referenced for biological assay. Each optimal concentration of antioxidants treated during aging *in vitro* maturation was approximately 20 times diluted BCX, 10 times diluted HES, and 600 times diluted ICA from each modified IC 50 value. Although HES-100 was more concentrated than the modified IC 50 value, BCX-1 treatment showed the best antioxidant activity when comparing the results from the chemical and biological assays. Together, BCX antioxidant is more effective and efficient at scavenging ROS compared with HES and ICA.

To measure reductive ability, the Fe^{3+}-Fe^{2+} transformation in the presence of different concentrations of antioxidants was investigated. Reducing power has previously been used as a good indicator of antioxidant activity as it represents the ability to reduce another substance by gaining electrons. For example, ROS can be reduced by antioxidants. Recently, the antioxidant capacities of four different kinds of microalgae were investigated using the DPPH scavenging activity, chelating activity, and reducing power assays. The study revealed that the polyphenol and carotenoid, but not flavonoid, contents of the microalgae were strongly correlated with antioxidant activity. Plant extracts with high amounts of carotenoid had the best antioxidant properties (Haoujar et al., 2019; Miceli et al., 2020; Taviano et al., 2018). The results from this study also demonstrated that BCX, a type of carotenoid, had a stronger reducing power than HES and ICA, both types of flavonoids, at the same μM concentration (Fig. 2A). This suggests that BCX has greater antioxidant activity than HES or ICA.

Although DPPH is an example of a ROS, it behaves differently to other more common free radicals in the biological environment. H_2O_2 is one of the most common ROS in the biological system and a major contributor to oxidative stress. Previously, the antioxidant capacity of coumarins was evaluated by means of a H_2O_2 scavenging assay (Al-Amiery, Al-

Majedy, Kadhum, & Mohamad, 2015). Vicilin was also evaluated for its antioxidant activity using the H_2O_2 scavenging assay at a specific concentration (Gupta, Srivastava, & Bhagyawant, 2018). Accordingly, we performed the H_2O_2 scavenging assay to compare the effect of three antioxidants at optimal concentration. The results indicated that approximately 5% of H_2O_2 was scavenged by the antioxidants compared with the control (Fig. 2B). The initial concentration of H_2O_2 solution was 4 mM, thus the final concentration after antioxidant scavenging was approximately 200 μM. Most studies previously used 200 μM of H_2O_2 to induce ROS damage. This suggests that the optimized concentrations of the antioxidants were sufficient to reduce oxidative stress induced by 200 μM of H_2O_2. Another study also showed that 0.1 μM of kaempferol significantly increased porcine blastocyst formation rate under oxidative stress induced by 200 μM of H_2O_2 (Yao et al., 2019). In our study, the H_2O_2 scavenging activity was not significantly different amongst the three antioxidants; however, even at lower concentrations, BCX-1 had a similar efficiency to HES-100 and ICA-5. Therefore, this result shows that BCX-1, HES-100, and ICA-5 have similar H_2O_2 scavenging activities compared with control.

Oxidative stress frequently occurs in aging post-ovulatory oocytes whereby the production of ROS is increased, and the protective abilities of antioxidants are decreased (Lord & Aitken, 2013). Intracellular GSH normally protects oocytes against oxidative stress induced by ROS; however, the levels of GSH decrease during the aging process (Boerjan & de Boer, 1990). Previously, Resveratrol treatment administered to goat oocytes was shown to significantly increase the levels of GSH, while allicin treatment administered to porcine oocytes significantly decreased the levels of ROS (Jeong et al., 2017; Piras et al., 2019). Our previous study showed that BCX, HES, and ICA can prevent oxidative stress during IVM of porcine oocytes at concentrations of 1, 100, and 5 μM, respectively (W. J. Kim et al., 2019; Park et al., 2018; Yoon et al., 2020). In this study, approximately 9% of ROS was generated

by aging oocytes, and BCX-1 was found to significantly eliminate approximately 16% of the ROS compared with HES-100 and ICA-5 (Fig. 3C). BCX-1 treatment recovered GSH to almost the same level as the controls.

The main function of *SOD1* is to catalyze the conversion of superoxide radicals into molecular oxygen and hydrogen peroxide (Del Rio, Sevilla, Gomez, Yanez, & Lopez, 1978). *SOD2* is the first line of defense against superoxide produced as a byproduct of oxidative phosphorylation (Li et al., 1995). Previously, Butylparaben treatment administered to mouse oocytes was shown to significantly increase the expression of *StAR*, *SOD1*, and *ERK1* (J. H. Kim & Jee, 2020). Additionally, Crocetin treatment administered to bovine oocytes was shown to significantly increase the expression of *SOD2*, *GPX1*, and *GPX4* (Dos Santos et al., 2019). Therefore, investigating the expression of the antioxidant genes (*SOD1* and *SOD2*) is a good indicator of antioxidant activity against oxidative stress. Taken together, our results suggest that BCX prevented oxidative stress in aging oocytes by decreasing the levels of ROS, promoting GSH, and increasing the expression of *SOD1* and *SOD2*.

The most common way to evaluate oocyte quality is to assess blastocyst formation rate, cell number, apoptotic rate, and the expression of important developmental and apoptotic genes (Luna et al., 2008). A recent study examined both the total cell number and proportion of apoptotic cells to check the quality of developing cat blastocysts during 6–9 days of *in vitro* culture (Ochota, Wojtasik, & Nizanski, 2016). Melatonin has also been shown to significantly increase the expression of developmental genes *POU5F1* and *SOX2* when administered to rabbit embryos (Mehaisen et al., 2015). In another study, the expression of anti-apoptotic genes (*BCL2L1* and *BIRC5*) was increased, while that of pro-apoptosis genes (*CASP3* and *FAS*) was decreased, when PC3 cells were treated with simvastatin (Goc et al., 2012). The *BCL2L1* protein belongs to the BCL-family to inhibit cell death by preventing the permeabilization of the mitochondrial membrane. BIRC5, also known as survivin, controls

apoptosis by inhibition of Bax and the extrinsic Fas pathways, and by binding to caspase-3 and −7 (Roman et al., 2016). The caspases are a family of protease enzymes that play essential roles in programmed cell death and inflammation. When procaspase-3 is activated by caspase 8, 9, or 10 (the initiators of apoptosis), caspase 3 performs mass proteolysis (Teshima, Ianez, Coutinho-Camillo, Tucker, & Lourenco, 2016). Fas is a cell-surface death receptor associated with FasL/Fas signaling. In the aging oocyte, apoptosis is initiated when the Fas ligand binds to the FAS receptor (Zhu et al., 2016). Our data showed that BCX-1 treatment significantly increased blastocyst formation rate and total cell number, and decreased the number of apoptotic cells. Further, BCX-1 treatment prevented apoptosis induced by *in vitro* maturation of aging oocytes by increasing the expression of development genes *POU5F1* and *SOX2*, and anti-apoptotic genes *BCL2L1* and *BIRC5*, and by decreasing the expression of the pro-apoptotic gene *CASP3,* compared with the aging control group. These results suggest that BCX treatment can improve the quality of blastocysts by preventing oxidative stress induced by oocyte aging.

In conclusion, this study suggests that BCX is a more powerful antioxidant than HES or ICA as determined by the DPPH, reducing power, and H_2O_2 scavenging activity assays. BCX had the lowest IC 50 value for inhibiting DPPH free radicals, and a stronger reducing power than HES or ICA at the same μM concentration. Although H_2O_2 scavenging activity was similar for all antioxidants, low concentrations of BCX-1 had equivalent H_2O_2 scavenging activity compared with HES-100 and ICA-5. BCX treatment was observed to improve porcine oocyte maturation, and enhanced blastocyst formation and development, thereby increasing the production of good-quality blastocysts for subsequent embryo development. Further, BCX protected against oxidative stress induced by oocyte aging increased the expression of antioxidant genes (*SOD1* and *SOD2*), developmental genes (*POU5F1* and *SOX2*), and anti-apoptotic genes (*BCL2L1* and *BIRC5*), and decreased the

expression of pro-apoptotic genes (*CASP3*). Taken together, the BCX antioxidant is more effective than HES or ICA to prevent ROS-mediated oxidative stress induced during oocyte aging. These findings could be further investigated for assisted reproductive technologies to improve embryonic viability.

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ABSTRACT IN KOREAN

본 연구에서는 β-크립토잔틴(BCX), 헤스페레틴(HES), 이카린(ICA) 항산화제 중 어떤 것이 돼지 난모세포의 체외 성숙(IVM)에 더 효과적인지를 조사하였다. 항산화 특성은 DPPH 소거능력, 환원력, H2O2 소거 능력 측정값을 비교하는 화학 분석방법과 노화된 돼지 난모세포와 배아를 이용하는 생물학적 분석방법으로 평가되었다. 화학 분석 결과는 BCX 가 대조군과 비교하여 HES 및 ICA 보다 DPPH 소거능력 및 환원력의 세기가 더 크다는 것을 증명했다. 그러나 항산화제의 H2O2 소거 능력은 1μM BCX (BCX-1), 100 μM HES (HES-100), 5 μM ICA (ICA-5) 의 최적 농도로 시험했을 때 모두 비슷하였다. 생물학적 분석실험 결과는 BCX-1 처리가 반응성 산소 종 (ROS) 의 유의적으로 시키고 글루타티온 수치를 향상시키며 항산화 유전자의 발현을 증가시키는데 더욱 효과적이라는 것을 보여주었다. 또한, BCX-1 은 단위발생된 배반포구에서 반독성 유전자들의 발현을 증가시키고, 친독성 유전자들의 발현을 감소시킴으로써 세포사멸을 억제했다. BCX-1 은 또한 노화 대조군인 HES-100 과 ICA-5 에 비해 배반포 형성률을 유의하게 증가시켰다. 본 연구는 난모세포의 노화 중 생성된 ROS 의 손상으로부터 체외성숙 배양액에 항산화제를 보충함으로써 예방될 수 있으며, BCX 는 보조 생식 기술을 개선할 수 있는 잠재적 후보일 수 있다는 것을 증명한다.

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