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Original Article

Effect of zeaxanthin on porcine embryonic development during *in vitro* maturation

Seo-jin Park[1,2], Kyoung-Ha So[1,2,✉], Sang-Hwan Hyun[1,2,✉]

[1] *Laboratory of Veterinary Embryology and Biotechnology, Veterinary Medical Center and College of Veterinary Medicine, Chungbuk National University, Seowon-gu, Cheongju 28644, Republic of Korea;*
[2] *Institute of Stem Cell & Regenerative Medicine, Chungbuk National University, Seowon-gu, Cheongju 28644, Republic of Korea.*

Abstract

Zeaxanthin is a common carotenoid, which is a powerful antioxidant that protects against damage caused by reactive oxygen species. The aim of the present study was to investigate the effects of zeaxanthin supplementation on *in vitro* maturation of porcine embryo development. We investigated nuclear maturation, intracellular glutathione (GSH), and reactive oxygen species (ROS) levels during in vitro maturation, and subsequent embryonic development following parthenogenetic activation and *in vitro* fertilization (IVF). The oocytes were maturated and used at the metaphase II stage. After 42 hours of *in vitro* maturation, the zeaxanthin-treated group (0.5 μmol/L) showed significant increases in nuclear maturation (89.6%) than the control group (83.4%) ($P < 0.05$). The intracellular GSH levels increased significantly ($P < 0.05$) as zeaxanthin concentrations increased; ROS generation levels decreased with increased zeaxanthin concentrations, but there were no significant differences. There were no significant differences in subsequent embryonic development, cleavage rate, blastocyst stage rate, and total blastocyst cell numbers following parthenogenetic activation and IVF when in vitro maturation media was supplemented with zeaxanthin. These results suggest that treatment with zeaxanthin during in vitro maturation improved the nuclear maturation of porcine oocytes by increasing the intracellular GSH level, thereby slightly decreasing the intracellular ROS level.

Keywords: *in vitro* maturation, porcine, embryonic development, zeaxanthin, antioxidant

Introduction

Porcine models, owing to their similarity to human anatomy, genetics, and physiology, have been used over many decades for biotechnology and biomedical applications to study specific disease resistance, xeno-transplantation, and therapeutics[1–2]. Therefore, in vitro production is very important and has been widely used thus far; however, the production of porcine embryos *in vitro* is more difficult than that of embryos obtained *in vivo*. A possible reason for this difficulty is an insufficient in vitro maturation (IVM) system. Several researchers have attempted to improve IVM conditions by the addition of microelements[3–4]. Microelements

✉Corresponding authors: Kyoung-Ha So, Ph.D., Laboratory of Veterinary Embryology and Biotechnology (VETEMBIO), College of Veterinary Medicine, Chungbuk National University, 1 Chungdae-ro, Seowon-gu, Cheongju 28644, Republic of Korea, Fax:+ 82-11-697-6520/+ 82-43-267-3150; Email:khso@cbu.ac.kr. Sang-Hwan Hyun, Ph.D., Professor, Laboratory of Veterinary Embryology and Biotechnology (VETEMBIO), College of Veterinary Medicine, Chungbuk National University, Chungdae-ro, Seowon-gu, Cheongju 28644, Republic of Korea, Fax:+ 82-10-6755-6088/+ 82-43-267-3150; Email:shhyun@cbu.ac.kr.

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possibly play important roles in IVM systems[5] and support female reproduction in many species[6–8]. Improved IVM systems would therefore facilitate more efficient embryo production and consequently contribute to greater biotechnological andmedical applications[9–12].

Zeaxanthin is a common carotenoid, which is a powerful antioxidant that is found in small amounts in the body, similarly to microelements. Zeaxanthin, lutein, and *meso*-zeaxanthin are xanthophyll carotenoids, and unlike other carotenoids, lutein and *meso*-zeaxanthin are constitutional isomers[13] and stereoisomers[14] of zeaxanthin, respectively, and owing to their structural similarity, they have similar physical properties[13]. Xanthophyll carotenoids (zeaxanthin and lutein) are predominantly located in the macular region of the retina[15–17] and accumulated in the liver, colon, pancreas, prostate, ovary, and lipophilic tissues, such as adipose tissue[18–21] in humans and other higher animals. Carotenoids protect against damage by reactive oxygen species (ROS) and macromolecules from oxidative damage[18,21–22]. Research related to humans, animals, and cell cultures has shown the potential role of zeaxanthin and lutein in preventing and protecting against several chronic disease, such as age-related macular degeneration and cataracts, cancer, specifically ovarian cancer, and coronary heart disease and strokes[23].

In the present study, we investigated the effects of zeaxanthin on porcine IVM and embryo development after parthenogenetic activation (PA) and in vitro fertilization (IVF), which evaluated nuclear maturation, intracellular levels of GSH and ROS, and embryonic developmental competence.

Materials and methods

Chemicals

All chemicals and reagents used in this study were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA), unless otherwise stated.

Oocyte collection and *in vitro* maturation

Ovaries of prepubertal gilts were collected at a local abattoir and transported to the laboratory within 2 hours in 0.9% (wt/vol) NaCl solution supplemented with 100 IU/L penicillin G and 100 mg/mL streptomycin sulfate at 32°C–35°C. The cumulus–oocyte complexes (COCs) in the ovaries were aspirated from 3 to 6 mm diameter superficial follicles using a disposable syringe with an 18-gauge needle. The COCs were washed with HEPES-buffered Tyrode's medium (TLH) containing 0.05% (wt/vol) polyvinyl alcohol (PVA) (TLH-PVA) and then 3 or more uniform layers of COCs were selected for IVM. Approximately 45 COCs were maturated in 500 μL TCM199 culture medium (TCM199; Invitrogen Corp., Carlsbad, CA, USA) supplemented with 0.6 mmol/L cysteine, 0.91 mmol/L sodium pyruvate, 10 ng/mL epidermal growth factor (EGF), 75 μg/mL kanamycin, 1 μg/mL insulin, 0.1% (vol/vol) PVA, 10 IU/mL equine chronic gonadotropin (eCG), and 10 IU/mL hCG (Intervet, Boxmeer, Netherlands), which were incubated at 39°C with 5% CO_2 in a 95% humidified chamber. Oocyte maturation was performed with (21–22 hours) or without (18–20 hours) hormones in IVM medium and the COCs were treated with or without zeaxanthin (0, 0.01, 0.05, 0.1 and 0.5 μmol/L) during the entire IVM, according to the experimental design.

Evaluation of nuclear maturation

The oocytes at the metaphase II (MII) stage, 40–42 hours after IVM, were sampled to analyze nuclear maturation. Samples of oocytes (606 oocytes were used for the nuclear maturation study) were denuded by gentle pipetting with 0.1% hyaluronidase in IVM medium and washed in TLH-PVA. The denuded oocytes were stained with 5 μg/mL Hoechst 33342 in TLH-PVA for at least 5 minutes. The stained oocytes were evaluated by fluorescence microscopy (Nikon Corp., Tokyo, Japan) with ultraviolet (UV) filters (330–385nm) at 400 × magnification, and classified as germinal vesicle (GV), metaphase I (MI), anaphase-telophase I (AT-I), or MII according to the meiotic maturation stage. The oocytes at MII were considered to have matured. The experiment was repeated three times.

Measurement of intracellular GSH and ROS levels

After IVM, the COCs were sampled 40–42 hours after IVM to determine intracellular GSH and ROS levels. The measurement of the GSH and ROS levels was performed according to previously described methods[24–25]. Briefly, 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CellTracker Blue; CMF2HC; Invitrogen Corp.) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen Corp., Paris, France) were used to detect intracellular GSH (blue fluorescence) and ROS levels (green fluorescence), respectively. From each treatment group, 8–9 oocytes were incubated in the dark for 30 minutes in TLH-PVA supplemented with 10 μmol/L CellTracker Blue and 10 μmol/L H_2DCFDA. After incubation, the oocytes were washed with TLH-PVA and the fluorescence was evaluated using an epifluorescence microscope (TE300; Nikon Corp.) with UV filters (370 nm for GSH and 460 nm for ROS) at 200 × magnification. The

fluorescence intensities of the oocytes were analyzed using Adobe Photoshop software (Version CS6; San Jose, CA, USA). The experiment was repeated thrice (GSH samples, $N = 25$; ROS samples, $n = 25$).

Parthenogenetic activation of oocytes

For PA, after 40–42 hours of IVM the COCs were denuded by gentle pipetting with 0.1% hyaluronidase, washed three times in TLH-PVA, and then rinsed with activation medium (280 mmol/L mannitol solution containing 0.01 mmol/L $CaCl_2$ and 0.05 mmol/L $MgCl_2$). For activation, the matured oocytes at the MII stage were placed between electrodes covered with activation medium in a chamber connected to an electrical pulsing machine (LF101; Nepa Gene, Chiba, Japan). Oocytes were activated with 2 direct-current pulses of 120 V/mm for 60 μseconds. After electrical activation, oocytes were immediately placed in *in vitro* culture (IVC) medium supplemented with 5 μg/mL cytochalasin B for 6 hours. The PA embryos were washed twice in fresh IVC medium, placed in 25 μL IVC droplets (10 gametes/drop) covered with pre-warmed mineral oil, and then cultured at 39°C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2 for 7 days. In all experiments, the culture media were renewed at 48 hours (day 2) after PA and 3 μL FBS was added to each drop at 96 hours (day 4) after PA. The experiment was repeated three times.

In vitro fertilization and culture

For IVF, matured oocytes were denuded with 0.1% hyaluronidase and washed in TLH-PVA. Groups of 15 matured oocytes were randomly placed in 40 μL droplets (15 gametes/drop) of modified Tris-buffered medium (mTBM) in a 35 × 10 mm Petri dish (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA) covered with pre-warmed mineral oil. Next, liquid semen supplied weekly from the Veterinary Service Laboratory (Department of Livestock Research, Republic of Korea) was stored at 17°C for 5 days before use. The semen sample was washed twice with Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.1% BSA via centrifugation at 2,000 *g* for 2 minutes. After washing, the sperm pellet was suspended in mTBM[26], which had been pre-equilibrated for 18 h at 39°C in 5% CO_2. After appropriate dilution, 5 μL of the sperm suspension was added to a 40 μL drop of fertilization medium (mTBM) to set the final sperm concentration at 1×10^6 sperm/mL. Immediately before fertilization, sperm motility was assessed, and more than 70% of motile sperm was used in each experiment. To use stored liquid semen, a modified two-step culture system[27] was used. The oocytes were co-incubated with the sperm for 20 minutes at 39°C in a humidified atmosphere of 5% CO_2 and 95% air. After 20 minutes of co-incubation with the sperm, the loosely attached sperm was removed from the zona pellucida via gentle pipetting. Next, the oocytes were washed twice in mTBM and incubated in mTBM without sperm for 5–6 hours at 39°C in a humidified atmosphere of 5% CO_2 and 95% air. Thereafter, the gametes were washed twice with embryo culture medium and cultured in 25 μL porcine zygote medium 3 (PZM3); each drop contained 10 gametes with pre-warmed mineral oil[28], which were incubated at 39°C for 168 hours (7 days) in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2. In all experiments, the culture media were replaced 48 hours (day 2) after IVF and 3 μL FBS was added to each drop at 96 hours (day 4) after IVF. The experiment was repeated three times.

Embryo evaluation and total blastocyst cell count

The day on which PA or IVF was performed was considered day 0. The embryos were evaluated for cleavage using a stereomicroscope on day 2 (48 hours), and were classified into three groups (2–3, 4–5, and 6–8 cells). On day 7, the number of blastocysts was counted and stained to determine the total cell number. Briefly, blastocysts were fixed with 1% paraformaldehyde and stained with 5 μg/mL Hoechst 33342 (bisbenzimide) for 5 minutes. Next, the blastocysts were mounted on glass slides in a drop of 100% glycerol, covered gently with a

Table 1 **Effect of zeaxanthin treatment on nuclear maturation during *in vitro* maturation**

Zeaxanthin concentration (μmol/L)	Oocytes cultured for maturation*	Number of oocytes			
		Germinal versicle(%)	Metaphase Ⅰ (%)	Anaphase and telophasei (%)	Metaphase Ⅱ (%)
0	126	4 (3.2±0.9)	13 (10.2±1.8)[a]	4 (3.2±0.8)	105 (83.4±0.9)[a]
0.01	121	6 (5.1±1.6)	9 (7.5±03)[a,b]	6 (4.9±2.5)	101 (83.4±0.9)[a]
0.05	120	3 (2.5±0.1)	11 (9.2±1.0)[a,b]	2 (1.6±0.8)	104 (86.7±0.9)[a,b]
0.1	122	4 (3.1±2.0)	13 (10.6±1.4)[a]	2 (1.5±0.8)	103 (84.6±2.6)[a]
0.5	117	4 (3.0±1.5)	6 (5.5±1.0)[b]	2 (1.9±1.1)	105 (89.6±0.3)[b]

Values with different superscript letters within a column are significantly different ($P < 0.05$). The data represents the mean±SEM.
* Experiment was replicated three times.

coverslip, and observed using a fluorescence microscope (Nikon) at 400 × magnification. The experiment was repeated thrice.

Experimental design

In Experiment 1, the effects of zeaxanthin supplementation during IVM on nuclear maturation were examined using various concentrations (0, 0.01, 0.05, 0.1, and 0.5 μmol/L). In Experiment 2, the effects of zeaxanthin treatment during IVM on intracellular levels of GSH and ROS were investigated. In Experiment 3, the effects of zeaxanthin treatment during IVM on subsequent embryonic development of PA and IVF embryos were examined.

Statistical analyses

The statistical analyses were performed using SPSS 12.0K software (SPSS, Inc., Chicago, IL, USA). Percentage data (e.g., rates of maturation, cleavage, blastocyst formation, and number of nuclei) were compared by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. All results are expressed as the mean±standard error of the mean (SEM). Probability values less than 0.05 ($P < 0.05$) were considered to indicate a statistically significant difference.

Results

Effects of zeaxanthin on nuclear and cytoplasmic maturation during in vitro maturation

MII oocytes were evaluated for nuclear maturation rates. There was no significant difference in maturation (MII stage) between the control (83.4%), 0.01 μmol/L (83.4%), and 0.1 μmol/L (84.6%) zeaxanthin groups, and no significant increase in the 0.05-μmol/L zeaxanthin group ($P>0.05$) (***Table 1***). However, the 0.5-μM zeaxanthin group (89.6%) showed a significantly increased number of MII stage oocytes than that of the control group ($P < 0.05$). In addition, the 0.5-μmol/L

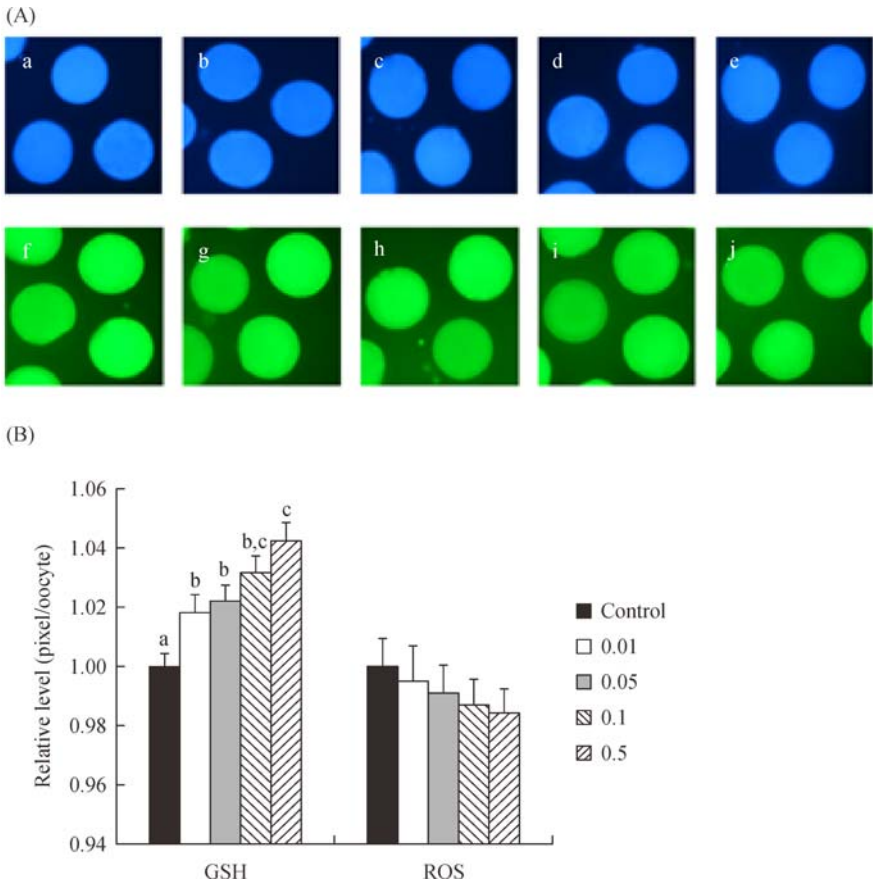

Fig. 1 **Epifluorescent photomicrographic images of *in vitro* matured porcine oocytes.** A: Oocytes were stained with CellTracker Blue (a–e) and 2', 7'-dichlorodihydrofluorescein diacetate (f– j) to detect intracellular levels of glutathione (GSH) and reactive oxygen species (ROS), respectively. Metaphase II oocytes derived from the maturation medium supplemented with 0.01 μmol/L (a and f), 0.05 μmol/L (b and g), 0.1 μmol/L (c and h), 0.5 μmol/L (d and i), or without zeaxanthin. B: Effect of zeaxanthin in maturation medium on intracellular GSH and ROS levels in *in vitro* matured porcine oocytes. Within each group (GSH and ROS) of end point, bars with different letters (a–c) are significantly different ($P < 0.05$). GSH samples, $n = 36$; ROS = 33. Experiment was replicated three times.

Table 2 **Effect of zeaxanthin treatment on embryonic development after parthenogenetic activation during *in vitro* maturation**

Zeaxanthin concentration (μmol/L)	No. of embryos cultured*	No. of embryos developed into(%)		Cell number in blastocyst**
		⩾2-cell embryos	Blastocysts	
0(Control)	90	46 (51.2±1.4)	30 (32.9±3.4)	100.8±6.9(19)
0.01	89	46 (51.4±2.0)	31 (35.4±5.6)	103.9±4.2(16)
0.05	95	54 (56.3±5.5)	42 (43.6±4.5)	103.6±3.8(21)
0.1	92	55 (59.1±4.6)	33 (35.9±2.5)	106.9±7.9(20)
0.5	99	56 (55.3±7.3)	33 (32.3±5.4)	108.5±1.2(17)

*Three replications. **Number of examined blastocysts.

zeaxanthin group had a significantly decreased number of MI oocytes (5.5%) than that of the control group (10.2%) ($P < 0.05$).

Effects of zeaxanthin in intracellular GSH and ROS levels during *in vitro* maturation

To assess cytoplasmic maturation, we examined intracellular GSH and ROS levels in MII oocytes derived from the maturation medium supplemented with zeaxanthin on IVM (***Fig. 1***). The intracellular GSH levels significantly increased as zeaxanthin concentrations decreased ($P < 0.05$). ROS generation levels decreased as zeaxanthin concentrations increased, but there was no significant difference.

Effects of zeaxanthin supplemented to in vitro maturation media on subsequent embryonic development following parthenogenetic activation and in vitro fertilization

Mature oocytes from each IVM group were subjected to PA to IVF. As shown in ***Table 2***, the PA embryos in the 0.1 μmol/L zeaxanthin-treatment group displayed the highest cell cleavage rates (56.3%), and the 0.05 μmol/L zeaxanthin-treatment group had the highest blastocyst stage rate (43.6%) compared to the other groups. The total cell number in blastocysts increased as zeaxanthin concentrations decreased, but all these groups were not significantly different. On day 2 after PA, there were the highest 4- to 5-cell PA embryos in the 0.1 μmol/L zeaxanthin-treatment group and the lowest 2- to 3-cell PA embryos in the 0.01 μmol/L zeaxanthin-treatment group, but all these results showed no significant differences (***Fig. 2***).

In the IVF experiment, the IVF embryos in the 0.05 μmol/L zeaxanthin-treatment group displayed the highest cleavage rates (50.5%), and the 0.1 μmol/L zeaxanthin-treatment group had the highest blastocyst stage rate (31.1%) than that of the other groups. The total cell number of blastocysts after IVF increased as the zeaxanthin concentration decreased, but all these groups showed no significant difference in statistical analysis (***Table 3***). On day 2 after IVF, the highest 2- to 3-cell IVF embryos were seen in the 0.5 μmol/L zeaxanthin-treatment group, 4- to 5-cell IVF embryos in the 0.1 μmol/L zeaxanthin-treatment group and 6- to 8-cell IVF embryos in the 0.01 μmol/L zeaxanthin-treatment group, but all of these result showed no significant differences (***Fig. 3***).

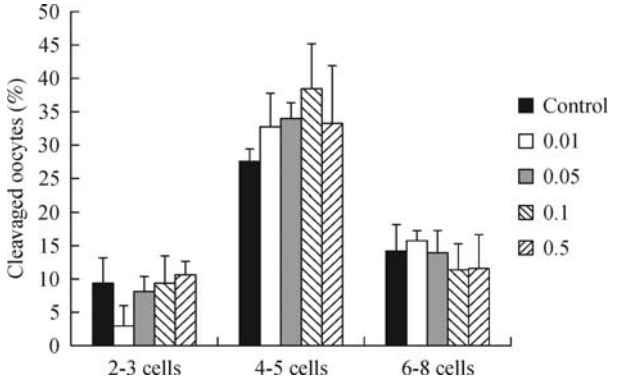

Fig. 2 **Effect of different concentrations of zeaxanthin treatment during *in vitro* maturation on cleavage pattern of parthenogenetic activation embryos on day 2.**

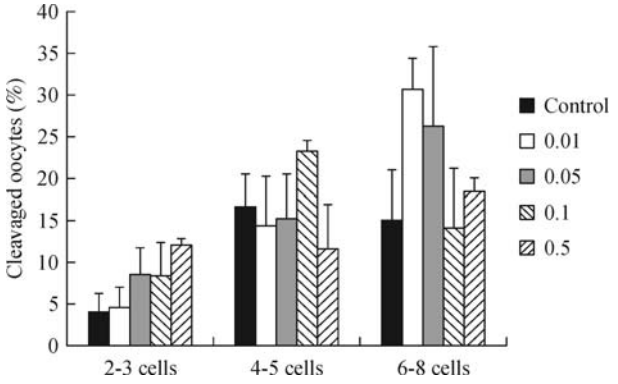

Fig. 3 **Effect of different concentrations of zeaxanthin treatment during *in vitro* maturation on cleavage pattern of *in vitro* fertilization embryos on day 2.**

Table 3 **Effect of zeaxanthin treatment on embryonic development after *in vitro* fertilization during *in vitro* maturation**

Zeaxanthin concentration (μM)	No. of embryos cultured*	No. of embryos developed into(%)		Cell number in blastocyst**
		⩾2-cell embryos	Blastocysts	
0	91	33 (36.2±0.9)	24 (26.4±0.6)	96.0±6.0(11)
0.01	91	46 (50.0±3.1)	21 (22.5±5.4)	95.7±3.3(11)
0.05	86	44 (50.5±3.3)	25 (30.0±5.1)	99.2±3.7(12)
0.1	94	43 (46.2±10.0)	29 (31.1±6.1)	100.±9.5(13)
0.5	98	41 (42.5±5.9)	15 (14.4±6.3)	117.1±13.0(7)

* Three replications.
** Number of examined blastocysts.

Discussion

Our results indicated that zeaxanthin treatment improved the nuclear and cytoplasmic maturation of porcine oocytes by increasing intracellular GSH levels and slightly decreasing ROS levels.

Although researchers have tried to improve the IVM of oocytes, the maturation efficiency of in vitro-matured oocytes is lower than that of *in vivo*-matured ones. It is particularly difficult to obtain high rates of fertilization and subsequent blastocyst development in vitro in pigs[29]. As oocyte maturation affects early embryonic development and survival, fetal growth and subsequent events[30–32], oocyte maturation is important in IVM systems. Thus, to improve the maturation of porcine oocytes matured in vitro, we supplemented the known antioxidant, zeaxanthin, in the present study.

Oocyte maturation includes both nuclear and cytoplasmic maturation. The level of intracellular GSH is a molecular marker of cytoplasmic maturation in mature porcine oocytes[24-33], and associated with various cellular processes, including protecting cells from oxidative damage[25] and regulating intracellular redox metabolism[34]. ROS are generated during the intermediate steps of oxygen reduction and can damage a variety of cellular structures at a high level. Oxidative stress in oocytes is an important parameter to evaluate oocyte health[35–36]. Zeaxanthin supplementation increased GSH levels and the GSH:GSSG ratio, particularly in response to oxidative stress[37].

Zeaxanthin scavenges superoxide, hydroxyl radicals, and inhibits tissue lipid peroxidation in a concentration-dependent manner *in vitro*[38]. In accordance with these studies, in the present study the intracellular GSH level increased as the zeaxanthin concentration increased, and the ROS level decreased, but there was no statistical difference. The zeaxanthin concentrations in the Henle fiber layer of the human macula are possibly between 0.1 and 1 mmol/L[13], and in these concentrations zeaxanthin exerts antioxidative effects. As we designed zeaxanthin treatment concentrations based on the human follicular fluid concentration[39], ROS decrease may be not significant. Although these data have shown that zeaxanthin may be a promising antioxidative agent during IVM, the mechanism that causes this antioxidative effect remains unclear. ROS can react with zeaxanthin by three different pathways (electron transfer, hydrogen abstraction, and radical addition), supported by *in vivo* and *in vitro* evidence, because of the zeaxanthin structure[13], and may exert antioxidant effects by more than one of these pathways. Further research is necessary to understand the mechanisms of the antioxidative effects.

In the present study, the nuclear maturation of MII stage oocytes increased significantly and MI stage oocytes decreased significantly in the high concentration zeaxanthin-treatment group (0.5 μmol/L) compared to that of the control group. These results suggest that zeaxanthin treatment enhanced the cytoplasmic and nuclear maturation of porcine oocytes. The embryonic development and blastocyst viability following PA and IVF showed no significant differences, although zeaxanthin affected oocyte maturation. These results could be due to a low zeaxanthin concentration as previously mentioned. Additional experiments at higher zeaxanthin concentrations should be conducted in further studies.

In the present study, zeaxanthin treatment during IVM enhanced cytoplasmic maturation by increasing intracellular GSH and decreasing ROS levels, and nuclear maturation by increasing MII stage oocytes. However, it is uncertain whether zeaxanthin-supplemented IVM medium affects subsequent in vitro development.

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