Effect of zeaxanthin on porcine embryonic development during in vitro maturation

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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, xenotransplantation, 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

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Zeaxanthin promotes porcine embryonic development

possibly play important roles in IVM systems⁵ and support female reproduction in many species⁶–⁸. Improved IVM systems would therefore facilitate more efficient embryo production and consequently contribute to greater biotechnological and medical applications⁹–¹².

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 isomers¹³ and stereoisomers¹⁴ of zeaxanthin, respectively, and owing to their structural similarity, they have similar physical properties¹³. Xanthophyll carotenoids (zeaxanthin and lutein) are predominantly located in the macular region of the retina¹⁵–¹⁷ and accumulated in the liver, colon, pancreas, prostate, ovary, and lipophilic tissues, such as adipose tissue¹⁸–²¹ in humans and other higher animals. Carotenoids protect against damage by reactive oxygen species (ROS) and macromolecules from oxidative damage¹⁸,²¹–²². 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 strokes²³.

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 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 matured 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% CO₂ 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 methods²⁴–²⁵. Briefly, 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CellTracker Blue; CMF2HC; Invitrogen Corp.) and 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA; 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 H₂DCFDA. 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 electrical pulsing machine (LF101; Nepa Gene, Chiba, Japan). For PA, after 40 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 droplet 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) 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 droplet at 96 hours (day 4) after PA. The experiment was repeated three times.

| Zeaxanthin concentration (μmol/L) | Oocytes cultured for maturation* | Number of oocytes | Germinal versicle(%) | Metaphase I (%) | Anaphase and telophase (%) | Metaphase II (%) |
|------------------------------------|--------------------------------|------------------|---------------------|----------------|---------------------------|----------------|
| 0                                  | 126                           |                  | 4 (3.2±0.9)        | 13 (10.2±1.8)  | 4 (3.2±0.8)               | 105 (83.4±0.9) |
| 0.01                               | 121                           |                  | 6 (5.1±1.6)        | 9 (7.5±0.3)\(^{ab}\) | 6 (4.9±2.5)               | 101 (83.4±0.9) |
| 0.05                               | 120                           |                  | 3 (2.5±0.1)        | 11 (9.2±1.0)\(^{ab}\) | 2 (1.6±0.8)               | 104 (86.7±0.9)\(^{ab}\) |
| 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)\(^{b}\) |

*Experiment was replicated three times.

VALUES WITH DIFFERENT SUPERScript LETTERS WITHIN A COLUMN ARE SIGNIFICANTLY DIFFERENT (P < 0.05). THE DATA REPRESENTS THE MEAN±SEM.

**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 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 droplet at 96 hours (day 4) after IVF. The experiment was repeated 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

![Epifluorescent photomicrographic images of in vitro matured porcine oocytes.](image-url)
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 2- to 3-cell PA embryos in the 0.1 μmol/L zeaxanthin-treatment group and 4- to 5-cell PA embryos in the 0.01 μmol/L zeaxanthin-treatment group, but all of 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 results showed no significant differences (Fig. 3).

**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.

![Fig. 2 Effect of different concentrations of zeaxanthin treatment during in vitro maturation on cleavage pattern of parthenogenetic activation embryos on day 2.](image1)

![Fig. 3 Effect of different concentrations of zeaxanthin treatment during in vitro maturation on cleavage pattern of in vitro fertilization embryos on day 2.](image2)
**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. As oocyte maturation affects early embryonic development and survival, fetal growth and subsequent events, 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, and associated with various cellular processes, including protecting cells from oxidative damage and regulating intracellular redox metabolism. 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. Zeaxanthin supplementation increased GSH levels and the GSH:GSSG ratio, particularly in response to oxidative stress.

Zeaxanthin scavenges superoxide, hydroxyl radicals, and inhibits tissue lipid peroxidation in a concentration-dependent manner. 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, and in these concentrations zeaxanthin exerts antioxidative effects. As we designed zeaxanthin treatment concentrations based on the human follicular fluid concentration, 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, 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 MI stage oocytes. However, it is uncertain whether zeaxanthin-supplemented IVM medium affects subsequent in vitro development.

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References

[1] Prather RS, Hawley RJ, Carter DB, et al. Transgenic swine for biomedicine and agriculture[J]. Theriogenology, 2003, 59(1): 115–123.

[2] Meurens F, Summerfield A, Nauwycck H, et al. The pig: a model for human infectious diseases[J]. Trends Microbiol, 2012, 20(1): 50–57.

[3] Jeon Y, Yoon JD, Cai L, et al. Supplementation of zinc on oocyte in vitro maturation improves preimplantation embryonic development in pigs[J]. Theriogenology, 2014, 82(6): 866–874.

[4] Kim E, Jeon Y, Kim DY, et al. Antioxidative effect of carboxyethylgermanium sesquioxide (Ge-132) on IVM of porcine oocytes and subsequent embryonic development after parthenogenetic activation and IVF[J]. Theriogenology, 2015, 84(2): 226–236.

[5] Mertz W. The essential trace elements[J]. Science, 1981, 213 (4514): 1332–1338.

[6] Córdova B, Morató R, Izquierdo D, et al. Effect of the addition of insulin-transferrin-selenium and/or L-ascorbic acid to the in vitro maturation of prepubertal bovine oocytes on cytoplasmic maturation and embryo development[J]. Theriogenology, 2010, 74(8): 1341–1348.

[7] Hostetler CE, Kincaid RL, Miranda MA. The role of essential trace elements in embryonic and fetal development in livestock [J]. Vet J, 2003, 166(2): 125–139.

[8] Favier AE. The role of zinc in reproduction. Hormonal mechanisms[J]. Biol Trace Elem Res, 1992, 32: 363–382.

[9] Banwell KM, Thompson JG. In vitro maturation of Mammalian oocytes: outcomes and consequences[J]. Semin Reprod Med, 2008, 26(2): 162–174.

[10] Walker SC, Shin T, Zaunbrecher GM, et al. A highly efficient method for porcine cloning by nuclear transfer using in vitro-matured oocytes[J]. Cloning Stem Cells, 2002, 4(2): 105–112.

[11] Mikkelsen AL. Strategies in human in-vitro maturation and their clinical outcome[J]. Reprod Biomed Online, 2005, 10(5): 593–599.

[12] Smitz JE, Thompson JG, Gilchrist RB. The promise of in vitro maturation in assisted reproduction and fertility preservation[J]. Semin Reprod Med, 2011, 29(1): 24–37.

[13] Krinsky NI, Landrum JT, Bone RA. Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye[J]. Annu Rev Nutr, 2003, 23: 171–201.

[14] Landrum JT, Bone RA. Lutein, zeaxanthin, and the macular pigment[J]. Arch Biochem Biophys, 2001, 385(1): 28–40.

[15] Bone RA, Landrum JT, Tarsis SL. Preliminary identification of the human macular pigment[J]. Vision Res, 1985, 25(11): 1531–1535.

[16] Bone RA, Landrum JT, Fernandez L, et al. Analysis of the macular pigment by HPLC: retinal distribution and age study [J]. Invest Ophthalmol Vis Sci, 1988, 29(6): 843–849.

[17] Handelman GJ, Dratz EA, Reay CC, et al. Carotenoids in the human macula and whole retina[J]. Invest Ophthalmol Vis Sci, 1988, 29(6): 850–855.

[18] Kaplan LA, Lau JM, Stein EA. Carotenoid composition, concentrations, and relationships in various human organs[J]. Clin Physiol Biochem, 1990, 8(1): 1–10.

[19] Schmitz HH, Poor CL, Wellman RB, et al. Concentrations of selected carotenoids and vitamin A in human liver, kidney and lung tissue[J]. J Nutr, 1991, 121(10): 1613–1621.

[20] de Lamirande E, Gagnon C. Reactive oxygen species (ROS) and reproduction[J]. Adv Exp Med Biol, 1994, 366: 185–197.

[21] Alafuf S, Heinrich U, Stahl W, et al. Dietary carotenoids contribute to normal human skin color and UV photosensitivity [J]. J Nutr, 2002, 132(3): 399–403.

[22] Rousseau EJ, Davison AJ, Dunn B. Protection by beta-carotene and related compounds against oxygen-mediated cytotoxicity and genotoxicity: implications for carcinogenesis and anticarcinogenesis[J]. Free Radic Biol Med, 1992, 13(4): 407–433.

[23] Ribaya-Mercado JD, Blumberg JB. Lutein and zeaxanthin and their potential roles in disease prevention[J]. J Am Coll Nutr, 2004, 23(6Suppl): 567S–587S.

[24] Abeydeera LR, Wang WH, Cantley TC, et al. Presence of beta-mercaptoethanol can increase the glutathione content of pig oocytes matured in vitro and the rate of blastocyst development after in vitro fertilization[J]. Theriogenology, 1998, 50(5): 747–756.

[25] Meister A. Selective modification of glutathione metabolism[J]. Science, 1983, 220(4596): 472–477.

[26] Abeydeera LR, Day BN. In vitro penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium[J]. Theriogenology, 1997, 48(4): 537–544.

[27] Gil MA, Ruiz M, Vazquez JM, et al. Effect of short periods of sperm-oocyte coincubation during in vitro fertilization on embryo development in pigs[J]. Theriogenology, 2004, 62(3-4): 544–552.

[28] Yoshioka K, Suzuki C, Tanaka A, et al. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium [J]. Biol Reprod, 2002, 66(1): 112–119.

[29] Gil MA, Cuello C, Parrilla I, et al. Advances in swine in vitro embryo production technologies[J]. Reprod Domest Anim, 2010, 45(Suppl 2): 40–48.

[30] Eppig JJ, O’Brien MJ. Comparison of preimplantation devel-
opmental competence after mouse oocyte growth and development in vitro and in vivo [J]. Theriogenology, 1998, 49(2): 415–422.

[31] Sirard MA, Richard F, Blondin P, et al. Contribution of the oocyte to embryo quality [J]. Theriogenology, 2006, 65(1): 126–136.

[32] Mtango NR, Potireddy S, Latham KE. Oocyte quality and maternal control of development [J]. Int Rev Cell Mol Biol, 2008, 268: 223–290.

[33] Wang WH, Abeydeera LR, Cantley TC, et al. Effects of oocyte maturation media on development of pig embryos produced by in vitro fertilization [J]. J Reprod Fertil, 1997, 111(1): 101–108.

[34] Luberda Z. The role of glutathione in mammalian gametes [J]. Reprod Biol, 2005, 5(1): 5–17.

[35] Yang HW, Hwang KJ, Kwon HC, et al. Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos [J]. Hum Reprod, 1998, 13(4): 998–1002.

[36] Halliwell B, Aruoma OI. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems [J]. FEBS Lett, 1991, 281(1-2): 9–19.

[37] Gao S, Qin T, Liu Z, et al. Lutein and zeaxanthin supplementation reduces H2O2-induced oxidative damage in human lens epithelial cells [J]. Mol Vis, 2011, 17: 3180–3190.

[38] Firdous AP, Preethi KC, Kuttan R. Antioxidant potential of meso-zeaxanthin a semi synthetic carotenoid[J]. Food Chem, 2010, 119(3):1096-1101.

[39] Schweigert FJ, Steinhagen B, Raila J, et al. Concentrations of carotenoids, retinol and alpha-tocopherol in plasma and follicular fluid of women undergoing IVF [J]. Hum Reprod, 2003, 18(6): 1259–1264.

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