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抗酸化効果に関する研究

The United Graduate School of Veterinary Science
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DO THI KIM LANH

LABORATORY OF VETERINARY MICROBIOLOGY
THE UNITED GRADUATE SCHOOL OF VETERINARY SCIENCE
YAMAGUCHI UNIVERSITY
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ABBREVIATION

ANOVA	analysis of variance
AT	anaphase I/ telophase I
BSA	Bovine serum albumin
CC	Condensed chromatin
COCs	Cumulus-oocyte complexes
Deg	Degenerated
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphat-buffered saline
eCG	Equine chorionic gonadotropin
EGF	Epidermal growth hormone
FBS	Fetal bovine serum
GSH	Glutathione
GV	Germinal vesicle
GVBD	Germinal vesicle break down
hCG	Human chorionic gonadotropin

HEPES	Hydroxyethyl-piperazineethane-sulfonic acid buffer
hMG	Human menopausal gonadotropin
Hoechst 33342	bisBenzimide H33342 trihydrochloride
ICSI	Intracytoplasmic sperm injection
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
MI	Metaphase I
MII	Metaphase II
mPBS	Modified phosphate-buffered saline
mSOF	Modified synthetic oviduct fluid
PBM	Porcine blastocyst medium
PFM	Porcine fertilization medium
PLSD	Protected least significant difference

PZM5	Porcine zygote medium 5
ROS	Reactive oxygen species
SCNT	Somatic cell nuclear transfer
TCM 199	tissue culture medium-199
TUNEL	terminal deoxynucleotidyl transferase nick-end labelling

SUMMARY

Recent studies have demonstrated that antioxidant supplementation could improve the *in vitro* embryo development in many species. The present studies were conducted to investigate the effects of sericin, melatonin, and astaxanthin on the development of porcine embryos when supplemented during *in vitro* maturation (IVM).

The first study aimed to examine the effects of sericin supplementation during *in vitro* oocyte maturation on the nuclear maturation, fertilization, and development of porcine oocytes. Cumulus-oocyte complexes (COCs) were cultured in maturation medium supplemented with 0 (control), 0.1, 0.5, 1.0, 2.5, or 5.0% sericin, and were then subjected to *in vitro* fertilization and embryo culture. More COCs matured with 1.0% sericin underwent germinal vesicle breakdown and reached the MII stage compared with the COCs matured without sericin (control, 92.9% and 65.2% vs 83.7% and 55.8%, $P < 0.01$). The proportions of oocytes with DNA-fragmented nucleus did not differ between the groups regardless of the sericin level. The total fertilization rate of oocytes matured with 1.0% sericin was higher ($P < 0.05$) than that of oocytes matured with 0.1%, 2.5%, and 5.0% sericin. Supplementation with more than 1.0% sericin decreased the DNA fragmentation index of the blastocysts compared with the control group ($P < 0.05$). However, the supplementation of the maturation medium with sericin had no beneficial effects on the cleavage, development to the blastocyst stage, and the total cell number of the embryos. Our findings indicate that supplementation with 1.0% sericin during maturation culture may improve the nuclear maturation and the quality of the embryos but does not affect blastocyst formation.

Melatonin has been reported to improve the *in vitro* development of embryos in some species. The present study was conducted to investigate the effect of melatonin supplementation during IVM and development culture on the development and quality of porcine embryos. In the first experiment, when the *in vitro*-fertilized embryos were cultured with different concentrations of melatonin (0, 10, 25, and 50 ng/ml) for 7 days, the blastocyst formation rate of embryos cultured with 25 ng/ml melatonin (10.7%) was significantly increased ($P < 0.05$) compared with the control embryos cultured without melatonin (4.2%). The proportion of DNA-fragmented nuclei in blastocysts derived from embryos cultured with 50 ng/ml melatonin was significantly lower ($P < 0.05$) than that of embryos cultured without melatonin (2.1% vs. 7.2%). In the second experiment, when oocytes were cultured in the maturation medium supplemented with different concentrations of melatonin (0, 10, 25, and 50 ng/ml), fertilized, and then cultured with 25 ng/ml melatonin for 8 days, there were no significant differences in the rates of cleavage and blastocyst formation among the groups. However, the proportions (2.7%-5.4%) of DNA-fragmented nuclei in the blastocysts derived from oocytes matured with melatonin were significantly decreased ($P < 0.05$) compared with those (8.9%) from oocytes matured without melatonin, irrespective of the concentration of melatonin. Our results suggest that supplementation of the culture media with melatonin (25 ng/ml) during IVM and development has beneficial effects on the developmental competence and quality of porcine embryos.

Heat stress can lead to a variety of disorders in reproductive functions such as impairment of oocyte maturation, fertilization, and embryonic development. Astaxanthin, one of the most common carotenoids, elicits antioxidant effects on the cellular viability and embryonic development. This study was conducted to investigate

the effects of astaxanthin on maturation, fertilization and development of porcine oocytes matured *in vitro* under heat stress conditions, and then fertilized and cultured under standard conditions. Porcine oocytes were cultured in maturation medium supplemented with different concentrations of astaxanthin (0, 0.25, 0.5 or 1 ppm) for 46 h at either 38.5 or 41 °C. In comparison to oocytes cultured at 38.5 °C, the exposure of porcine oocytes to 41.0 °C during IVM significantly inhibited their maturation and development of fertilized oocytes to the blastocyst stage. Supplementation of maturation medium with astaxanthin (0.5 ppm) significantly improved oocyte maturation, fertilization and development to the blastocysts stage in both experimental groups. However, the total cell number and the apoptosis index of the blastocysts did not differ among the groups. Moreover, astaxanthin (0.5 ppm) significantly increased the rate of oocytes reached to the MII stage and decreased proportion of apoptotic oocytes exposed to H_2O_2 (1.0 mM) during IVM. In summary of this experiment, we demonstrated that supplementation of the maturation medium with astaxanthin (0.5 ppm) exerted antioxidative effects and improved the ability of maturation, fertilization, and development of porcine oocytes exposed to heat stress.

In conclusions, our findings indicated that the supplementation of a kind of antioxidants during *in vitro* maturation or development has significant benefits on the developmental competence of the porcine embryos by reducing oxidative stress, which is a major detrimental effect on *in-vitro* culture system. Supplementation with either 1.0% sericin, 25 ng/ml melatonin, or 0.5 ppm astaxanthin has particularly beneficial effects on oocyte maturation, fertilization, and their development to the blastocyst stage. Astaxanthin may be further effective for the protection of porcine oocytes that are exposed to heat stress or H_2O_2.

GENERAL INTRODUCTION

Pig is a common research model

The IVP of mammalian embryos includes three major technical steps: IVM, IVF, and IVC of fertilized oocytes. The porcine IVP embryo system is of great interest to produce large quantities of matured oocytes and embryos that are used not only for basic sciences such as physiology and reproduction, but also for advanced biotechnology and biomedical research.

The use of animal models in human health and diseases as well as in biomedical research has been increasingly recognized since the completion of animal genome sequences has been discovered. Compared with mouse models, which have certainly contributed to our understanding of gene function, the pig shows greater similarity to humans in both genome sequence as well as anatomy and physiology, thus making it a superior comparative model (Critser et al. 2009). In recent years, pigs are considered as one of the excellent animal models providing a suitable source of cells and organs for xenotransplantation (Ramsoondar et al. 2009, Luo et al. 2012, Prather et al. 2013), and as transgenic animals to produce the specific proteins relevant to human biology (Takahagi et al. 2005, Pan et al. 2010, Tanihara et al. 2016).

However, the developmental competence of IVP embryos in pigs is lower than that of their in vivo counterparts as well as of other mammalian species such as cattle or mice (Kikuchi et al. 1999, Dang-Nguyen et al. 2011). Thus, improving the competence

of IVP embryos is crucial because the inefficiency of IVP system diminishes its application in further studies as these techniques depend on blastocysts as their material source.

In vitro embryo production in pigs

In comparison to *in vivo* embryo production, IVP in pigs has several advantages. It enables us to utilize a significant number of follicular oocytes, immature oocytes in the other words, that are normally lost through atresia by using slaughterhouse ovaries as the basic material for oocyte collection. Thus, the IVM of oocytes provides matured oocytes at the MII stage that can be used as recipient oocytes for other reproductive technologies such as ICSI (Nakai et al. 2003) and cloning (Betthauser et al. 2000, Onishi et al. 2000, Boquest et al. 2002). These technologies are now used to generate transgenic pigs (Kurome et al. 2006, Cho et al. 2009, Watanabe et al. 2015).

Moreover, IVP makes it possible to produce a larger number of embryos at lower costs and in shorter time. These advantages could increase their application to further studies since these techniques depend on zygotes or blastocysts as source materials. As recently confirmed by Tanihara et al. (2016), the use of gene editing by electroporation applied to zygotes has the potential for facilitating genetic modification of pigs because this technology does not require the complicated methods associated with manipulators as in somatic reprograming or ISCI. The improvement in IVP systems may manifest its efficacy in pork production by creating new transgenic species with better body and meat characteristics or growth.

In vitro reproduction in pigs started when the ability of porcine IVM oocytes to be fertilized was first reported by Motlik and Fulka (1974). The first successful IVP embryos in pigs were reported four years later by Iritani et al. (1978). Important steps toward success were made when Nagai et al. (1988) successfully used frozen-thawed boar spermatozoa for IVF. The developmental competence to the blastocyst stage of IVF embryos after IVM was first confirmed by Mattioli et al. (1989). Further, piglets were born after IVP embryo transfer cultured to the 2–4 cell stage (Yoshida et al. 1993) or to the morula stage (Abeydeera et al. 1998). However, the first successful instances of embryos transfer at the blastocyst stage were reported about 15 years ago (Marchal et al. 2001, Kikuchi et al. 2002).

Despite the improvements in many aspects of IVF procedures, porcine IVP still struggles with problems that remained unsolved over the years, such as insufficient cytoplasmic ability for development, polyspermy of IVM oocytes, and improper culture conditions for IVP embryos (Nagai et al. 2006). These problems detain other reproductive techniques such as SCNT, ICSI, embryo transfer, and establishment and utilization of embryonic stem cells, which are essential for the production of transgenic animals, and also cells and organs for xenotransplantation. Thus, improving the competence of IVP embryos is crucial because the inefficiency of the IVP system diminishes its application on further studies since these techniques depend on blastocysts as source of materials.

Objectives of the study

Along with inadequate culture medium, IVP embryos have low developmental competence in terms of blastocyst formation rate and quality (measured as cell number in blastocysts). Moreover, IVP blastocysts show high incidences of DNA fragmentation and apoptotic cells. For the success of porcine IVP, it is essential to produce normal embryos. The normalcy of porcine IVP embryos is highly impaired by either high incidence of abnormal chromosome numbers caused by polyspermic fertilization of oocytes arrested at a diploid stage (Kikuchi et al. 2009), or the imperfection of culture systems to maintain or support the developmental competence of embryos (Dang-Nguyen et al. 2011).

Moreover, it is well-known that the oxygen concentration within the lumen of the female reproductive tract is about one third of that found under standard *in vitro* conditions (Mastroianni and Jones 1965). In general, the high oxygen concentration associated with *in vitro* conditions, results in the generation of increased reactive oxygen species (ROS) and in turn, increased oxidative stress (Agarwal et al. 2003, Agarwal et al. 2006), which induces apoptosis (Kannan et al., 2000).

Therefore, ensuring embryo quality by improving the status of matured oocytes, developing IVF systems with high frequencies of monospermic fertilization and establishing a proper culture medium, seem to be the desirable approaches to solve these problems. Ensuring embryo quality in IVP systems also includes the improvement of their viability and developmental competence, which may be possible by further efforts to reduce the detrimental effects of culture systems, such as by reducing oxidative stress or by development of new media specifically designed for pigs. The detrimental effects

of oxidative stress can be effectively reduced by the application of low oxygen tension during embryo culture (Karja et al. 2004) or by the application of antioxidants to the medium (Ozawa et al. 2006).

In this study, we focus on supporting oocytes/embryos by reducing oxidative stress, which is a major detrimental effect of the *in vitro* culture system on the developmental competence of porcine embryos. This study contains following three Studies;

Study 1: Effect of sericin supplementation during *in vitro* maturation on the maturation, fertilization, and development of porcine oocytes,

Study 2: Melatonin supplementation during *in vitro* maturation and development supports the development of porcine embryos

and

Study 3: Astaxanthin present in the maturation medium reduces the negative effect of heat shock on the developmental competence of porcine oocytes.

CHAPTER I:

Study 1: EFFECT OF SERICIN SUPPLEMENTATION DURING IN VITRO MATURATION ON THE MATURATION, FERTILIZATION AND DEVELOPMENT OF PORCINE OOCYTES

INTRODUCTION

The IVP techniques for porcine embryos have been improved, but the development rate of embryos derived from *in vitro* maturation and fertilization to the blastocyst stage and their quality are still low compared with those of in vivo-derived embryos. The low developmental competence of porcine IVP embryos might be caused in part by suboptimal conditions for culture of oocytes and/or embryos (Funahashi and Day 1997). Earlier studies have demonstrated that the mediation of antioxidants to prevent damage to porcine oocytes caused by oxidative stress plays an important role in the acquisition of developmental competence after fertilization (Tatemoto et al. 2001, Whitaker and Knight 2010).

Sericin is a water-soluble component of silk that covers fibroin fibres and fixes them to each other in cocoons. Sericin has an antioxidant effect in skin fibroblast cells exposed to hydrogen peroxide and has the potential to inhibit the production of intracellular hydrogen peroxide in keratinocytes treated with ultraviolet B irradiation (Dash et al. 2008a, 2008b). At present, however, no information is available concerning the applications of silk proteins to the *in vitro* culture of porcine oocytes or embryos.

The Study 1 was conducted to examine whether the supplementation of the *in vitro* maturation medium with sericin would improve the nuclear maturation, fertilization, and development of porcine oocytes.

MATERIALS AND METHODS

Unless otherwise indicated, all chemicals were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA).

IVM, IVF, and IVC were carried out according to the method described by Namula et al. (Namula et al. 2013). COCs from porcine ovaries obtained at a slaughterhouse were cultured for 22 h in IVM medium without β-mercaptoethanol. The COCs were then transferred to IVM medium without hormones and were subsequently cultured for an additional 22 h. All of the incubations were performed in a humidified incubator containing 5% CO_2 in air at 38.5°C. To examine the effects of sericin supplementation during maturation culture on the meiotic competence, fertilization ability, and development of oocytes, the COCs were cultured in the IVM medium supplemented with 0 (control), 0.1, 0.5, 1.0, 2.5, and 5.0% (w/v) sericin (Wako Pure Chemical Industries Ltd., Osaka, Japan).

After maturation culture, the oocytes were co-incubated with frozen-thawed spermatozoa (5×10^6 cells/ml) for 20 h in a 38.5°C humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. The zygotes were subsequently cultured in droplets of PZM-5 (Research Institute for the Functional Peptides Co., Yamagata, Japan) in a 38.5°C humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. Seventy-two hours after insemination, all of the cleaved embryos were transferred into fresh PZM-5 with 5% fetal bovine serum and cultured for an additional five days to evaluate their ability to develop to the blastocyst stage.

The meiotic stage and the DNA damage of oocytes, and the total cell number and DNA fragmentation in the blastocysts were analysed using a combined technique for simultaneous nuclear staining and terminal deoxynucleotidyl transferase nick-end labelling (TUNEL) that was previously described by Wongsrikeao et al. (2004) and Isobe et al. (2012). To assess the fertilization status of the oocytes, some presumptive zygotes were stained with acetic orcein (Namula et al. 2013).

The statistical significance was inferred from ANOVA followed by Fisher's PLSD test using STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). All of the percentage data were subjected to arcsin transformation before the statistical analysis. Differences with a probability value (P) of 0.05 or less were regarded as significant.

RESULTS

As shown in Table 1.1, more COCs matured with 1.0% sericin underwent GVBD and reached the MII stage compared with the control COCs that matured without sericin ($P < 0.01$). No significant differences were found in the proportions of oocytes with DNA-fragmented nuclei between the groups, regardless of the sericin concentration.

As shown in Table 1.2, the total fertilization rate of the oocytes matured with 1.0% sericin was higher ($P < 0.05$) than those of the oocytes matured with 0.1%, 2.5%, and 5.0% sericin. The fertilization rate of oocytes matured with 1.0% sericin tended to be higher ($P < 0.1$) than that of the control oocytes cultured without sericin. The rate of

development to the blastocyst stage of the oocytes matured with 1.0% sericin was higher ($P < 0.05$) than those of the oocytes matured with 0.1% and 5.0% sericin. The supplementation of the IVM medium with more than 1.0% sericin had no beneficial effects on the total cell number of blastocysts (Fig. 1.1A) but decreased the DNA fragmentation index of the blastocysts ($P < 0.05$) compared with the control oocyte group (Fig. 1.1B).

DISCUSSION

Our findings show that the supplementation of the IVM medium with 1.0% sericin improves the meiotic competence of the oocytes and the quality of the blastocysts determined by the DNA fragmentation index. To the best of our knowledge, the current study is the first to report the effects of sericin supplementation during maturation culture on the meiotic competence of porcine oocytes. It has been demonstrated that oxidative stress during IVM culture induces the apoptotic cell death of porcine oocytes (Tatemoto et al. 2000). We recently reported that sericin can prevent oxidative stress during bovine embryo culture and thus results in the improvement of the embryo quality and increased embryonic development (Isobe et al. 2012). In the present study, the supplementation of the IVM culture with sericin exerted a promoting effect on the nuclear maturation of porcine oocytes. However, there were no differences in the proportions of oocytes with DNA-fragmented nuclei between the sericin-supplemented groups and the control group. In the present study, we matured the porcine oocytes under normal (20%) oxygen tension in TCM199 medium supplemented

with 0.6 mM cysteine and without β-mercaptoethanol to reduce the number of antioxidants. Cysteine is a critical amino acid component of GSH, and cysteine supplementation during maturation culture increases the GSH concentration in the oocytes (Viet Linh et al. 2009). As a major non-protein thiol, GSH plays an important role in the protection of cells against oxidative stress (Meister 1983), particularly when the cells are cultured under high oxygen tension. Therefore, the lack of a positive effect of sericin supplementation on DNA damage in the oocytes may be partly due to the increase in GSH concentration in the oocytes by cysteine added to the IVM medium in our system.

In the present study, the highest fertilization rate was observed in the oocytes matured with 1.0% sericin, and this number tended to be higher than the number of control oocytes cultured without sericin. Although the supplementation of the IVM medium with sericin had no beneficial effects on cleavage, development to the blastocyst stage, and total cell number of embryos, supplementation with more than 1.0% sericin decreased the DNA fragmentation index of the blastocysts. Compared with previous studies conducted with other oxidants such as N-acetyl-cysteine and N-acetyl-cysteine-amide or ascorbic acid 2-O-alpha-glucoside (Tatemoto et al. 2001, Whitaker and Knight 2010), the effects of sericin on the blastocyst formation rate was less potent. Although this discrepancy remains to be explained, the difference may reflect the variation in the culture system, such as the culture medium, culture conditions, and the presence of hormones and growth factors. In this study, moreover, IVF and IVC were performed under a low (5%) oxygen tension. Therefore, the supplementation of culture medium with sericin during IVM may not be enough to observe differences in the

development and quality of embryos, and the oocytes or embryos may need a continuous exposure to a high oxygen concentration (20%) during the *in vitro* culture.

In conclusion, our findings indicate that supplementation of 1.0% sericin during maturation culture has a slight effect on the nuclear maturation and fertilization of porcine oocytes and the quality of the embryos but does not affect blastocyst formation. Further studies are necessary to evaluate the exact role and mechanism of sericin on porcine oocyte maturation and embryonic development.

CHAPTER II:

Study 2: MELATONIN SUPPEMENTATION DURING IN VITRO MATURATION AND DEVELOPMENT SUPPORTS THE DECVELOPMENT OF PORCINE EMBRYOS

INTRODUCTION

The IVP of porcine embryos is a valuable tool that aids the development of assisted reproductive technologies. However, the development rate of embryos derived from IVM and IVF and their quality are still low compared with those of *in vivo*-derived embryos (Gil et al. 2010, Dang-Nguyen et al. 2011). The low developmental competence of porcine IVP embryos could be partially due to the suboptimal culture conditions for the oocytes or embryos (Funahashi and Day 1997). Many studies have shown negative effects of ROS on cellular functions, such as damage to proteins, lipids, and nucleic acid components, and this damage can result in mitochondrial alterations (Guerin et al. 2001), the two-cell block of embryos (Nasr-Esfahani and Johnson 1992), and reduced embryo development (Watson et al. 1994, Blondin et al. 1997b). The addition of antioxidants to culture medium has been shown to reduce intercellular H_2O_2 and increase the developmental competence of porcine embryos (Kitagawa et al. 2004, Suzuki and Yoshioka 2005, Suzuki et al. 2007).

Melatonin (N-acetyl-5-methoxytryptamine), a pineal secretory product, is a free radical scavenger, antioxidant, and anti-apoptotic factor (Hardeland 2005). In comparison to other free radical scavengers, melatonin is multifunctional and universal (Hardeland 2005). Because of its solubility in water and lipids, melatonin acts as a hydrophilic and hydrophobic antioxidant. A previous study demonstrated that the supplementation of media with melatonin improves the development rate of fertilized mouse embryos (Ishizuka et al. 2000). In sheep, melatonin supplementation increased the rates of development to the hatched blastocyst stage of frozen-thawed blastocysts (Abecia et al. 2002). Moreover, melatonin supplementation during maturation culture

promotes the meiotic competence of porcine oocytes and the development of parthenogenetic embryos (Kang et al. 2009). In contrast, it has been reported that supplementation with melatonin during IVF has no beneficial effects on the development of bovine embryos (Cheuqueman et al. 2015). Moreover, the presence of melatonin has been demonstrated to have no positive effects on the blastocyst formation rate of porcine oocytes after IVF (Rodriguez-Osorio et al. 2007). Therefore, it is not fully understood whether melatonin supplementation during oocyte maturation and embryo culture positively affects the development of porcine IVF embryos.

The objective of the Study 2 was to determine whether melatonin supplementation during *in vitro* maturation and development culture improves the development and quality of porcine IVF embryos.

MATERIALS AND METHODS

Oocyte preparation and *in vitro* maturation

Porcine ovaries were obtained from a slaughterhouse and transported to the laboratory within 3 h in physiological saline (0.9% (w/v) NaCl) at 30°C. The ovaries were washed three times with modified phosphate-buffered saline (m-PBS; Nihonzenyaku, Fukushima, Japan) supplemented with 100 IU/ml penicillin G potassium (Meiji, Tokyo, Japan) and 0.1 mg/ml streptomycin sulfate (Meiji). The COCs were collected from 3- to 6-mm follicles using a surgical blade. Only COCs with a uniform dark-pigmented ooplasm and an intact cumulus cell mass were collected. Approximately 50 COCs were then cultured in 500 μl of maturation medium consisting

of 25 mM HEPES tissue culture medium 199 with Earle's salts (TCM 199; Invitrogen Co., Carlsbad, CA, USA) supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma-Aldrich), 50 μM sodium pyruvate (Sigma-Aldrich), 2 mg/ml D-sorbitol (Wako Pure Chemical Industries Ltd.), 1 μg/ml 17 β-estradiol (Sigma-Aldrich), 10 IU/ml equine chorionic gonadotropin (Kyoritu Seiyaku, Tokyo, Japan), 10 IU/ml human chorionic gonadotropin (Kyoritu Seiyaku), and 50 μg/ml gentamicin (Sigma-Aldrich) for 22–24 h in 4-well dishes (Nunc A/S, Roskilde, Denmark). Subsequently, the COCs were transferred to maturation medium without hormone supplementation and cultured for an additional 22 h according to the method previously described by Namula et al. (Namula et al. 2013). The incubations of COCs were performed in a 38.5°C humidified incubator containing 5% CO_2.

IVF and embryo culture

IVF was performed according to the method described by Namula et al. (Namula et al. 2013), with minor modifications. Frozen-thawed spermatozoa were transferred into 5 ml of fertilization medium (PFM; Research Institute for the Functional Peptides Co.) in a 15-ml test tube and were then washed by centrifugation at 500 × *g* for 5 min. The pellet of spermatozoa was resuspended in the fertilization medium to obtain a final sperm concentration of 1×10^7 cells/ml. The spermatozoa (50 μl) were introduced into 50 μl of fertilization medium containing 10-20 matured oocytes. The final sperm concentration was adjusted to 5×10^6 cells/ml. The oocytes were co-incubated with the spermatozoa for 20 h in a 38.5°C humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. After co-incubation with spermatozoa, the

inseminated oocytes were denuded from the cumulus cells and the attached spermatozoa by mechanical pipetting.

The denuded oocytes were subsequently transferred to 100-μl droplets of PZM-5 (Research Institute for the Functional Peptides Co.). Each droplet contained approximately 10 presumed zygotes. They were cultured continuously *in vitro* in a 38.5°C humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. At 72 h after insemination, all of the cleaved embryos were transferred into 100-μl droplets of PZM-5 supplemented with 5% fetal bovine serum (Hyclone, Thermo Fisher Scientific Inc., Logan, UT, USA) and cultured for an additional five days to evaluate their ability to develop to the blastocyst stage.

To evaluate DNA fragmentation in the blastocyst, the blastocysts were fixed on day 7 (day 0; insemination) and were analyzed using a combined technique for simultaneous nuclear staining and TUNEL modified from the procedures previously described by Otoi et al. (1999). In brief, the blastocysts were fixed overnight at 4°C in 3.7% (w/v) paraformaldehyde diluted in Dulbecco's PBS (DPBS; Invitrogen Co.). After fixation, the blastocysts were permeabilized in DPBS containing 0.1% (v/v) Triton-X100 for 40 min. Then they were subsequently incubated overnight at 4°C in DPBS containing 10 mg/ml bovine serum albumin (A9647, Sigma-Aldrich). The blastocysts were then incubated in fluorescein-conjugated 2′-deoxyuridine-5′-triphosphate and TUNEL reagent (Roche Diagnostics Corp., Tokyo, Japan) for 1 h at 38.5°C. After TUNEL staining, the embryos were counterstained with 1 μg/ml DAPI (Invitrogen Co.) for 10 min. They were then treated with an anti-bleaching solution (Slow-Fade; Molecular Probes Inc., Eugene, OR, USA), mounted on a glass slide, and sealed with

clear nail polish. The labeled blastocysts were then examined using a microscope (Eclipse 80i, Nikon, Tokyo, Japan) fitted with epifluorescence illumination. The proportion of DNA-fragmented nuclei in a blastocyst was calculated by dividing the number of cells containing fragmented DNA in their nuclei by the total number of cells.

Experiment 1

To determine the effects of melatonin supplementation during IVC on the development to blastocysts of presumed zygotes and on the quality of the blastocysts, the zygotes were cultured in culture medium supplemented with 0, 10, 25 or 50 ng/ml of melatonin (Sigma-Aldrich). The concentrations of melatonin used for Experiment 1 were chosen basing on a study by Kang et al. (Kang et al. 2009), where supplementation with 10-50 ng/ml melatonin during IVM culture improved the meiotic competence of porcine oocytes and the development of parthenogenetic embryos.

Experiment 2

The concentration of melatonin (25 ng/ml) that was found to be most suitable for the blastocyst formation rates during the IVC of the zygotes in Experiment 1 was used in this experiment. To examine the effects of melatonin supplementation during IVM culture on the development to blastocysts of oocytes after IVF, oocytes were cultured in IVM medium supplemented with 0, 10, 25 or 50 ng/ml of melatonin. After IVM, the oocytes were fertilized and then cultured with 25 ng/ml melatonin.

Statistical analysis

All of the experiments were performed six or seven times. Statistical significance was inferred from ANOVA followed by Fisher's PLSD test using STATVIEW (Abacus Concepts, Inc.). All percentage data were subjected to arc sin transformation before statistical analysis. Differences at a probability value (p) of 0.05 or less were considered to be significant.

RESULTS

The effects of melatonin supplementation during IVC on the development and quality of embryos are shown in Table 2.1. There were no significant differences in the cleavage rates among the groups. However, the blastocyst formation rate of the embryos cultured with 25 ng/ml melatonin (10.7%) was significantly higher ($P < 0.05$) than that of the embryos cultured with 10 ng/ml melatonin and without melatonin (4.3 and 4.2%, respectively). The proportion of DNA-fragmented nuclei in the blastocysts derived from embryos cultured with 50 ng/ml melatonin (2.1%) was significantly lower ($P < 0.05$) that that of the embryos cultured without melatonin (7.2%).

The effects of melatonin supplementation during IVM on the development and quality of the embryos are shown in Table 2.2. There were no significant differences in the rates of cleavage (74.2–86.4%) or blastocyst formation (11.0–15.0%) among the groups. The proportions (2.7–5.4%) of DNA-fragmented nuclei in the blastocysts derived from the oocytes matured with melatonin were significantly lower ($P < 0.05$) compared with those (8.9%) of the oocytes matured without melatonin, irrespective of the concentration of melatonin. Moreover, the proportion of DNA-fragmented nuclei in

the blastocysts derived from oocytes matured with 25 ng/ml melatonin was significantly lower ($P < 0.05$) than that from oocytes matured with 50 ng/ml melatonin.

DISCUSSION

It has been suggested that the use of melatonin as an antioxidant during culture of oocytes/embryos can improve the development of porcine embryos (Kang et al. 2009, Shi et al. 2009). Melatonin is a scavenger of nitric oxide radicals, and one of its metabolites inhibits nitric oxide synthase activity and thus influences oxygen consumption in embryos (Noda et al. 1999, Leon et al. 2006). In the present study, our findings showed that when porcine oocytes were cultured in the maturation medium supplemented with melatonin, the supplementation of melatonin improved the quality of embryos. In particular, the supplementation with 25 ng/ml of melatonin reduced the proportions of DNA-fragmented nuclei in the blastocysts. These results are in agreement with the previous experiment (Tian et al. 2014) reporting that melatonin supplementation at suitable concentrations promoted the meiotic competence of oocytes and the development and quality of embryos in cattle. They suggested that melatonin supplementation during IVM significantly upregulated the expressions of oocyte maturation associated genes and cumulus cells. In contrast, (Nakano et al. 2012) reported that supplementation of melatonin during IVM did not significantly improve the maturation rates of porcine oocytes or the blastocyst formation rates after parthenogenetic activation. Moreover, they demonstrated that melatonin treatment during IVC reduced the levels of reactive oxygen species in parthenogenetic embryos

but did not reduce the proportion of apoptotic cells in parthenogenetic blastocysts. Kang et al. (2009) also reported that melatonin-treated oocytes during IVM have significantly lower levels of ROS than the control (untreated) oocytes, but melatonin treatment during IVM did not improve the cleavage rates or blastocyst cell number. In this study, supplementation with melatonin during IVC did not affect the cleavage rates or the total cell number (46.1–59.3 cells) of blastocysts (data not shown). However, supplementation with melatonin (25 ng/ml) during IVC increased the blastocyst formation rates, and supplementation with 50 ng/ml of melatonin reduced the proportions of DNA-fragmented nuclei in the blastocysts compared with blastocysts that did not receive melatonin. Moreover, supplementation with melatonin during both IVM and IVC did not increase the rates of cleavage and blastocyst formation, but it did improve the quality of blastocysts, irrespective of the concentrations of melatonin present during IVM. Our results indicate that the effects of melatonin supplementation on embryonic development may differ depending on the supplementation period. For example, supplementation with melatonin during IVC may improve the developmental competence of embryos, and the positive effects of melatonin on the quality of embryos becomes apparent by the supplementation during IVM.

In conclusion, we demonstrated that supplementation with melatonin during embryo culture has beneficial effects on the developmental competence of embryos, and the supplementation with melatonin during both IVM and IVC improves the quality of embryos. The free radical scavenging potency and anti-apoptotic activity of melatonin may be responsible for these observed effects.

CHAPTER III:

Study 3: ASTAXANTHIN PRESENT IN THE MATURATION MEDIUM REDUCES NEGATIVE EFFECTS OF HEAT SHOCK ON THE DEVELOPMENTAL COMPETENCE OF PORCINE OOCYTES

INTRODUCTION

Reproductive performance in livestock is generally affected by climatic conditions (Gwazdauskas 1985, Sieuve-de-Menezes 2011). Heat stress is a crucial factor that can disrupt reproductive processes by altering the regulation of body temperature (Hansen 2009). Adverse climatic conditions, such as elevated temperature and heat indices have been shown to elicit significant effects on litter size, farrowing rate and weaning to first service interval in Yorkshire and Landrace sows (Tantasuparuk et al. 2000). Heat stress has been demonstrated to significantly reduce conception rates in gilts exposed to higher ambient temperature during the early period of implantation (Omtvedt et al. 1971). Moreover, compared with other species, porcine oocytes exhibited an increased sensitivity not only to low but also to elevated temperatures (Barati et al. 2008).

Roth and Hansen (2004) suggested that activation of apoptotic processes mediated by group II of caspases, which is caused by heat stress during bovine oocyte maturation, is a critical mechanism responsible for the disruption of oocyte capacity to support early embryonic development. It has been suggested that heat reduces intracellular concentration of the antioxidant GSH in embryos and that the addition of various antioxidants to culture media, including taurine, GSH, and vitamin E, provide some thermoprotection of embryos (Hansen and Arechiga 1999). Moreover, dietary supplementation with antioxidants such as vitamins C and E protected oocytes against aged-associated disturbances in segregation of chromosomes during maturation (Tarin et al. 1998).

Astaxanthin is a common carotenoid that is extracted from fishery products

(Miki et al. 1982). Recent studies have reported that astaxanthin exhibits the ability to scavenge not only most reactive oxygen species (ROS) but also the hydroxyl radical (Hama et al. 2012). As a scavenger of peroxyl radicals, astaxanthin is twice as effective as β-carotene in liposomes (Goto et al. 2001) and exceeds in quenching of reactive oxygen in *E. coli* compared with β-carotene and vitamin E (Tatsuzawa et al. 2000). It has been demonstrated that astaxanthin improved the development of *in vitro* bovine embryos exposed to heat stress by reducing the expression of the mRNA of Src homology 2 domain-containing transforming protein C1 (SHC1), an oxidative stress adaptor protein, and superoxide dismutase 2 (SOD2), a mitochondrial reactive oxygen species (ROS) scavenger (Namekawa et al. 2010). However, limited information concerning the effects of astaxanthin on IVM of oocytes and early embryonic development is currently available for pigs.

We hypothesized that astaxanthin supplementation during *in vitro* maturation protects porcine oocytes from the deleterious effects of heat stress and improves the development of oocytes after maturation culture. Therefore, in the Study 3**,** we investigated the effects of astaxanthin supplementation during IVM on the ability of maturation, fertilization, and further embryonic development of porcine oocytes exposed to 41.0 °C. Hydrogen peroxide has been used to induce oxidative stress because its cellular effects and chemical reactivity have been well studied. Therefore, we evaluated antioxidant effects of the astaxanthin treatment on the meiotic competence and apoptosis of porcine oocytes exposed to hydrogen peroxide during IVM.

MATERIALS AND METHODS

IVM and assessment of meiotic status

Porcine ovaries were obtained from 6- to 7-month-old peripubertal crossbred gilts (Landrace × Large White) at a local slaughterhouse and transported to the laboratory within 3 h in physiological saline (0.9% (w/v) NaCl) at 30 °C. Ovaries were washed three times with the modified phosphate-buffered saline (m-PBS; Nihonzenyaku) supplemented with 100 IU/mL penicillin G potassium (Meiji) and 0.1 mg/mL streptomycin sulfate (Meiji). The COCs were collected from follicles by slicing the ovarian surface using a surgical blade (about 15 COCs per ovary). Only COCs with uniformly dark-pigmented ooplasm and intact cumulus cell masses were collected. Approximately 50 COCs were cultured in 500 μL maturation medium, consisting of 25 mM HEPES tissue culture medium 199 with Earle's salts (TCM 199; #12340, Invitrogen Co.) supplemented with 10% (v/v) porcine follicular fluid from ovarian follicles of peripubertal crossbred gilts, 0.6 mM cysteine (Sigma-Aldrich), 50 μM sodium pyruvate (Sigma-Aldrich), 2 mg/mL D-sorbitol (Wako Pure Chemical Industries Ltd.), 1 μg/mL 17β-estradiol (Sigma-Aldrich), 10 IU/mL equine chorionic gonadotropin (Kyoritu Seiyaku), 10 IU/mL human chorionic gonadotropin (Kyoritu Seiyaku), and 50 μg/mL gentamicin (Sigma-Aldrich), for 22–24 h in 4-well dishes (Nunc A/S). Subsequently, the COCs were transferred into the maturation medium without hormone supplementation and cultured for an additional 22 h according to the method previously described (Namula et al. 2013). All incubations were performed in a humidified incubator containing 5% CO_2 in air.

To assess meiotic status of oocytes after IVM, about 25% of the COCs were denuded, fixed and permeabilized in Dulbecco's PBS (DPBS; Invitrogen Co.) supplemented with 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton X-100 (Sigma-Aldrich) at room temperature for 15 min. Permeabilized oocytes were then placed on glass slides and stained with 1.9 mM bisbenzimide (Hoechst 33342; Sigma-Aldrich) before being overlaid on with coverslips. The oocytes were examined by fluorescence microscopy after an incubation overnight at 4 °C. They were classified according to their chromatin configuration as “GV”, “GVBD”, “MI”, “AT” or “MII” (Wongsrikeao et al. 2004) (Fig. 3.1A-E). Oocytes displaying unidentifiable chromatin configurations were classified as “degenerated” (Fig. 3.1F).

IVF and IVC

The IVF was performed according to a method described by Namula et al. (2013) with minor modifications. Frozen-thawed spermatozoa from a Large White fertile boar, aged 1.5 years were transferred into 5 mL of fertilization medium (PFM; Research Institute for the Functional Peptides Co.) in a 15-mL test tube and were then washed by centrifugation at 500×*g* for 5 min. The pellets of spermatozoa were resuspended in fertilization medium to obtain a final sperm concentration of 1×10^7 cells/mL. A part of the spermatozoa (50 μL) was introduced into 50 μL of fertilization medium containing 10-20 mature oocytes under a layer of mineral oil (Sigma-Aldrich) in a 35×10 mm petri dish (Falcon; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The final sperm concentration was adjusted to 5×10^6 cells/mL. The oocytes were co-incubated with spermatozoa for 20 h in a 38.5 °C humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. After the co-incubation with spermatozoa for

20 h, the inseminated oocytes were denuded from the cumulus cells and the attached spermatozoa by mechanical pipetting.

To assess the fertilization rates of the oocytes, about 25% of denuded zygotes were mounted on glass slides and fixed with acetic acid: ethanol (1:3 v/v) for 48-72 h. The fixed zygotes were stained with acetic orcein (1% orcein in 45% acetic acid) and examined by a phase contrast microscopy. Oocytes containing both female and male pronuclei were considered as fertilized and were categorized as monospermic or polyspermic according to the number of swollen sperm heads and pronuclei in the cytoplasm (Fig. 3.2).

Embryo culture and assessment of blastocyst quality

The remaining denuded zygotes were subsequently transferred to 100-μL droplets of porcine zygote medium (PZM-5; Research Institute for the Functional Peptides Co.) under a layer of mineral oil. Each droplet contained approximately 10 presumed zygotes. The zygotes were cultured continuously *in vitro* at 38.5 °C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. At 72 h after insemination, all cleaved embryos were transferred into 100-μL droplets of porcine blastocyst medium (PBM; Research Institute for the Functional Peptides Co.) and cultured for an additional five days to evaluate their ability to develop to the blastocyst stage.

To evaluate the total cell number and apoptosis in the blastocysts, a total of 157 embryos at the early and expanded blastocyst stages were fixed on day 7 (day 0; insemination) and were analyzed using a combined technique for simultaneous nuclear and TUNEL staining modified from procedures previously described (Otoi et al. 1999).

Briefly, the blastocysts were fixed overnight at 4°C in 3.7% (w/v) paraformaldehyde diluted in PBS. After fixation, they were permeabilized in PBS containing 0.1% (v/v) Triton-X100 for 40 min. They were subsequently incubated overnight at 4 °C in PBS containing 10 mg/mL bovine serum albumin (blocking solution). They were then incubated in fluorescein-conjugated 2-deoxyuridine 5-triphosphate and TUNEL reagent; (Roche Diagnostics Corp., Tokyo, Japan for 1 h at 38.5 °C. After TUNEL staining, the embryos were counterstained with 1 µg/mL DAPI (Invitrogen Co.) for 10 min. They were then treated with an anti-bleaching solution (Slow-Fade; Molecular Probes Inc.), mounted on glass slides, and sealed with clear nail polish. The labeled blastocysts were examined using an epifluorescence microscope (Eclipse 80i, Nikon). Apoptotic nuclei showed a condensed and fragmented morphology (Brison and Schultz 1997, Pawlak et al. 2011) (Fig. 3.3). The apoptotic index was calculated by dividing the number of cells containing apoptotic nucleus (labelled by TUNEL) by the total number of cells.

Experiment 1

To evaluate the effects of astaxanthin supplementation during IVM on oocyte maturation, fertilization, and the blastocyst formation rate, oocytes were matured under heat stress, in maturation medium supplemented with 0, 0.25, 0.5, and 1 ppm astaxanthin (Fuji Chemical Industry Co., Ltd, Toyama, Japan) at 38.5 or 41.0 °C. The concentrations of astaxanthin used in Experiment 1 were established based on by (Namekawa et al. 2010) who reported that supplementation of 0.25 ppm astaxanthin improved the development of bovine embryos exposed to heat stress.

Experiment 2

We assessed the protective effect of astaxanthin on H_2O_2-induced apoptosis in porcine oocytes. In Experiment 1, we found that astaxanthin at concentration of 0.5 ppm had beneficial effects on the maturation, fertilization and the blastocyst formation rate in oocytes exposed to heat stress during IVM. In a preliminary experiment, we confirmed that the exposure of 1 mM H_2O_2 induces apoptosis in porcine oocytes (data not shown). Therefore, the COCs were exposed to 1 mM H_2O_2 in maturation medium supplemented with or without astaxanthin (0.5 ppm) during IVM. After culture at 38.5 °C for 46 h, the oocytes were fixed and evaluated for nuclear status and apoptosis using the TUNEL technique. Oocytes with apoptotic nucleus (labeled by TUNEL) was classified as apoptotic oocyte.

Statistical analysis

The following parameters were statistically analyzed: the number of oocytes that reached the MII stage (maturation rate), number of fertilized oocytes (total fertilization rate), number of monospermic oocytes (monospermic fertilization rate), cleavage rate, blastocyst formation rate, total cell count within a blastocyst and apoptotic index**.** The examined parameters were analyzed by ANOVA using the general linear models (GLM) procedure of SAS (SAS for Windows, version 9.1, SAS Institute, Cary, NC, USA). The statistical model included the culture temperature, concentration of astaxanthin and two-way interactions. The maturation rate and the proportion of apoptotic oocytes were analyzed using a statistical model that included H_2O_2 exposure, astaxanthin supplementation and two-way interactions. When significant interactions were not observed between the two parameters, they were excluded from the model.

The differences with a probability value of $P \leq 0.05$ were considered as statistically significant.

RESULTS

Effects of astaxanthin on the maturation and fertilization of porcine oocytes exposed to heat stress during IVM

The maturation rate of control oocytes (no astaxanthin) exposed to 41.0 °C was reduced ($P < 0.05$) compared with that of oocytes cultured at 38.5 °C (16.5% vs. 47.9%) (Table 3.1). However, there were no differences in the total and monospermic fertilization rates between the oocytes cultured at 38.5 and 41.0 °C (33.3% vs. 25.2%, and 61.1% vs. 83.3%, respectively).

When oocytes were matured at 38.5 °C, the maturation rates of oocytes cultured with 0.5 and 1.0 ppm astaxanthin were higher ($P < 0.05$) compared with the control oocytes (69.4% and 67.0% vs. 47.9%). Irrespective of its concentration, astaxanthin also significantly increased the total fertilization rate of oocytes. When oocytes matured at 41.0 °C, the supplementation of astaxanthin increased ($P < 0.05$) the maturation rates (0.5 and 1 ppm) and total fertilization rates (only 0.5 ppm) of the treated oocytes compared with controls (52.4% and 50.5% vs. 16.5%, and 54.9% vs. 25.2%, respectively).

Effects of astaxanthin on the development and quality of embryos derived from porcine oocytes exposed to heat stress during IVM

In the control oocytes cultured without astaxanthin, the blastocyst formation rate in oocytes exposed to 41.0 °C was lower ($P < 0.05$) compared with oocytes cultured at 38.5 °C (0.9% and 10.7%, respectively) (Table 3.2). However, the cleavage rate, total cell number and apoptotic index did not differ between control blastocysts derived from oocytes exposed to 41.0 and 38.5 °C.

When oocytes matured at 38.5 °C, the cleavage rate of oocytes cultured with 0.25 ppm astaxanthin was higher ($P < 0.05$) than that of oocytes cultured with 0 or 0.5 ppm astaxanthin (79.5% vs. 68.1 or 68.6%). The blastocyst formation rate of oocytes cultured with all doses of astaxanthin was higher ($P < 0.05$) than those of control oocytes. There were no differences in the total cell numbers or apoptotic index among the groups. When oocytes matured at 41.0 °C, the cleavage rate of oocytes cultured with 0.5 ppm astaxanthin was higher ($P < 0.05$) than those from other oocyte groups**.** Moreover, astaxanthin increased ($P < 0.05$) the blastocyst formation rate of oocytes exposed to 41.0 °C but did not improve the total cell number and apoptotic index.

Effects of astaxanthin on the maturation rates and proportions of apoptotic oocytes exposed to oxidative stress during IVM

The astaxanthin supplementation increased ($P < 0.05$) the maturation rate of oocytes exposed to H_2O_2 as well as cultured without H_2O_2 (Fig. 3.4A). The proportion of oocytes with apoptotic nucleus was higher ($P < 0.05$) in oocytes exposed to H_2O_2 during IVM compared with the non-exposed oocytes. However, astaxanthin reduced ($P < 0.05$) the proportion of oocytes with apoptotic nucleus (Fig. 3.4B).

DISCUSSION

A limited number of studies reported that, during the early preimplantation stage (up to the eight-cell), porcine and bovine embryos are more vulnerable to heat stress compared with those during the more advanced stages (Omtvedt et al. 1971, Sakatani et al. 2004). In the present study, we focused on the effects of elevated temperature exerted during oocyte maturation but not during the early embryonic development. We confirmed that heat stress (41 °C) during IVM of porcine oocytes negatively affects their maturation rate. These results are consistent with our previous findings (Barati et al. 2008), demonstrating that acute (1 h) exposure of porcine oocytes to an elevated temperature (41 °C) decreased the maturation rate of oocytes. The increased incidence of apoptosis and cytoskeletal disruption in the oocytes after direct exposure to elevated temperature before or during IVM was previously reported in cattle and pigs (Roth and Hansen 2005, Ju and Tseng 2004). In contrast, the exposure of bovine oocytes to elevated temperature during maturation did not affect the fertilization rate, but reduced the blastocyst formation rate (Payton et al. 2004, Ju et al. 1999). Similarly, in this study, it can be demonstrated that exposure of porcine oocytes to elevated temperature during their maturation did not reduce the total and monospermic fertilization rates but decreased the blastocyst formation rate. The results are consistent with those of Tseng et al. (2006) who found that exposure of porcine oocytes to thermal stress (41.5 °C) for 2 h reduced the development to the blastocyst stage of parthenogenetic embryos but did not influence the apoptosis index in the embryos. Payton et al. (2004) and Ju et al. (1999)

reported that heat stress during IVM altered the redistribution of cortical granules in oocytes, coincident with reduced blastocyst development. However, the stress elicited few effects on fertilization or functional competence of the ooplasm to remodel sperm nuclei. Taken together, the results of our experiments emphasized the direct impact of elevated temperature on the meiotic competence of porcine oocytes and, in consequence, on the reduction of the blastocyst rate formation.

It was suggested that apoptosis maintains cellular quality in the inner cell mass (ICM) of the blastocyst by eliminating damaged cells or those expressing inappropriate phenotype or developmental incompetence (Hardy 1997). An increase in the incidence of apoptosis of the blastocysts developed *in vitro* was suggested to be attributable to the absence of the maternal factors that under *in vivo* conditions function as survival factors (Brison and Schultz 1997). Moreover, heat stress during oocyte maturation promoted an apoptotic response mediated by group II of caspases (Roth and Hansen 2004, Chang and Yang 2000). Therefore, apoptosis level of the oocytes and embryos is a useful indicator of the oocyte quality and embryonic development (Brison and Schultz 1997, Tatemoto et al. 2000).

It has been demonstrated that supplementation of culture medium with antioxidants protects oocytes and embryos from the deleterious effects of heat and oxidative stress (Caamano et al. 1998, Maya-Soriano et al. 2013). Astaxanthin represents one of the most common carotenoids and it elicits antioxidant effects on cellular viability and embryonic development (Jang et al. 2010). The antioxidative effects of astaxanthin on embryonic development have been suggested to be attributed to the induction of antioxidant genes and the suppression of apoptotic genes (Jang et al. 2010). In this study, we found that when oocytes were exposed to H_2O_2 during IVM,

astaxanthin effectively restored the oocyte maturation rate as well as protected oocytes from apoptosis. The mechanism underlying the damage induced by H_2O_2 during IVM involves the generation of ROS, which induces apoptosis in oocytes. Therefore, our results confirmed the antioxidant effects of astaxanthin on H_2O_2-treated oocytes during IVM.

Namekawa et al. (2010) reported that astaxanthin abolished the impairment of embryo development caused by heat stress. In this study, we found that supplementation of maturation medium with astaxanthin (0.5 ppm) improved the oocyte maturation and fertilization regardless the temperature (38.5 or 41 °C). This is consistent with previous reports demonstrating that antioxidants (ascorbic acid and cysteine) prevented porcine oocytes from damage caused by oxidative stress, and that they played an important role in the acquisition of developmental competence after fertilization (Tatemoto et al. 2001, Whitaker and Knight 2010). Although the quality of embryos did not differ among the treatment groups, the improvement in the blastocyst formation rate resulting from astaxanthin supplementation during IVM was observed in oocytes cultured both at 38.5 and 41.0 °C, irrespective of the astaxanthin concentration. In contrast, Namekawa et al. (2010) reported that astaxanthin exerted beneficial effects on the development of bovine embryos in the heat stressed group but not in the control group. In this study, to reduce the number of reagents, we subjected porcine oocytes to IVM under normal (20%) oxygen tension in TCM199 medium supplemented with 0.6 mM cysteine but without β-mercaptoethanol. It appears that astaxanthin positively affects embryonic development in the oocytes cultured at 38.5 °C. Moreover, our results indicate that the beneficial effect of astaxanthin on the maturation and subsequent development of oocytes subjected to heat stress might be exerted *via* antioxidative effects.

Dietary supplementation with antioxidants such as vitamins C and E, carotenoids, and selenium can eliminate ROS-induced damage and maintain normal cellular functions (Chew and Park 2004). Moreover, a supplemental intake of vitamins C and E decreases the risk for the ovulation of aneuploid and diploid oocytes in aged female mice (Tarin et al. 1998). Hansen et al. (2001) reported that dietary supplementation with astaxanthin improved the number of corpora lutea, implantation sites and fetuses in minks. Therefore, the use of astaxanthin as a dietary supplement during heat stress may enhance the fertility by the improvement of oocyte maturation and development.

In conclusion, we demonstrated that heat stress (41 °C) during IVM of porcine oocytes negatively influenced maturation and development of embryos after IVF, however**,** astaxanthin supplementation during IVM protected oocytes from deleterious effects of heat stress. Astaxanthin at concentration of 0.5 ppm was particularly effective in the improvement of maturation, fertilization, and development of oocytes exposed to heat stress during IVM in pigs.

GENERAL DISCUSSION AND CONCLUSION

The history of *in vitro* reproduction in pigs began four decades ago (Motlik and Fulka 1974). Despite the application of various modifications to improve the quality of resultant embryos, the developmental competence of IVP embryos in pigs is still low compared with that of their in vivo counterparts as well as to *in vitro* development of other species such as cattle or mice (Kikuchi et al. 1999, Dang-Nguyen et al. 2011). On the other hand, the IVP system for porcine embryos is of tremendous interest to produce large quantities of matured oocytes and embryos that are critically used not only for basic sciences such as physiology and reproduction, but also for advance biotechnology and biomedical research. Because of the number of anatomic and physiologic similarities with humans, the pig is considered as a suitable source of cells and organs for xenotransplantation (Critser et al. 2009, Ramsoondar et al. 2009) as well as a transgenic animal to produce the specific proteins that it shares with human biology (Takahagi et al. 2005, Pan et al. 2010). Improving the developmental competence of porcine IVP embryos is essential for the application of further studies in human health.

The oxygen concentration in the standard *in vitro* system was found to be higher than that within the female reproductive tract (Mastroianni and Jones 1965). Moreover, the high oxygen concentration associated with *in vitro* conditions results in increased ROS generation, and in turn, increases oxidative stress that has been reported to have a negative impact on embryo quality and may also lead to early embryonic development blockade and retardation (Agarwal et al. 2003, Agarwal et al. 2006). Several evidences indicate that supplementation of the culture medium with antioxidants, vitamins C and

E, amino acids, or ROS scavengers can be an alternative treatment strategy to reduce oxidative stress and provide benefits such as embryo survival and blastocyst formation rates in animal studies reviewed in Taylor (2001).

In Study 1, it can be indicated that supplementation with 1.0% sericin during maturation culture has a slight effect on the meiotic competence of oocytes and the quality of blastocysts as determined by the DNA fragmentation index, but does not affect blastocyst formation ability. In Study 2, supplementation with melatonin during embryo culture has beneficial effects on the developmental competence of embryos, and supplementation with melatonin during both IVM and IVC improves the quality of embryos. Astaxanthin supplementation during IVM protected oocytes from the deleterious effects of heat stress. In Study 3, astaxanthin at a concentration of 0.5 ppm was particularly effective in improving maturation, fertilization, and the development of oocytes exposed to heat stress during IVM in pigs. When oocytes were exposed to H_2O_2 during IVM, astaxanthin effectively restored the oocyte maturation rate as well as protected oocytes from apoptosis. Hydrogen peroxide is a ROS member that is more stable than O_2 and can readily diffuse and pass through cell membranes. A direct relationship between increased H_2O_2 concentration and apoptosis was observed in fragmented embryos and blastocysts in mouse and human (Yang et al. 1998, Lee and Yeung 2006). H_2O_2 was also considered as a mediator of apoptosis in blastocysts (Pierce et al. 1991). Moreover, oocyte maturation and embryo development are also affected by increased ROS or decreased antioxidant defences in bovine (Blondin et al. 1997a, Harvey et al. 2002). Therefore, the apoptosis level of oocytes and embryos is a useful indicator of oocyte quality and embryonic development (Brison and Schultz 1997,

Tatemoto et al. 2000). In this study as well, we confirmed the deleterious effects of H_2O_2 on inducing apoptosis in porcine oocytes matured *in vitro*.

Oxidative stress is a certain threat to oocytes and embryos *in vitro* since these cells are removed from their natural habitat, which is lack of maternal antioxidant factors. Consequently, oxidative stress significantly affects the competence of IVP system outcomes. However, *in vitro* embryos also require oxygen at low concentrations for their further development (Quinn and Harlow 1978). It is widely accepted that the optimal incubation atmosphere for the ICV of porcine embryos is under 5% CO_2 and 5% O_2 (Berthelot and Terqui 1996, Kikuchi et al. 2002), whereas a variety of media has been developed for the porcine IVP system. Each media system requires a balance between oxygen factors and antioxidants. Therefore, it is necessary to investigate the optimal concentration of the supplemented antioxidants. In this study, we have found that supplementation with 1.0% sericin during IVM (Study 1), 25 ng/ml of melatonin (Study 2), or 0.5 ppm astaxanthin (Study 3) was particularly beneficial to oocyte maturation, fertilization, and subsequently to their development to the blastocyst stage. Astaxanthin was further effective in the protection of porcine oocytes that are exposed to heat stress or H_2O_2 from apoptosis.

Although our data are limited, they do allow us to suggest that the supplementation of a kind of antioxidants during *in vitro* maturation or development has significant benefits on the developmental competence of the porcine embryos by reducing oxidative stress, which is a major detrimental effect on *in vitro* culture system. In many attempts for supporting the development of porcine IVP system, our finding might be a desirable approach that benefit for the application of further *in vitro* studies in pig. On the other hand, previous studies have demonstrated that dietary

supplementation with antioxidants such as vitamins C and E, carotenoids, and selenium can eliminate ROS-induced damage and maintain normal cellular functions (Chew and Park 2004). Moreover, a supplemental intake of vitamins C and E decreases the risk for the ovulation of aneuploid and diploid oocytes in aged female mice (Tarin et al. 1998). Hansen et al. (2001) reported that dietary supplementation with astaxanthin improved the number of corpora lutea, implantation sites and fetuses in minks. According to the University of Maryland Medical Center, melatonin also regulates the start of menstruation, the length of ovulation cycles and menopause. Therefore, the use of antioxidant as a dietary supplement may enhance the fertility and reproductive performance by the improvement of oocyte maturation and development. In addition, the antioxidants using in our study are natural source antioxidants, which will be safe for using as dietary supplementation for farm animal.

TABLES AND FIGURES

Table 1.1. Effects of sericin supplementation in the maturation medium on the meiotic competence of porcine oocytes*

Concentration of sericin (%)	No. of oocytes examined	No. (%) of oocytes with**		No. (%) of oocytes with DNA-fragmented nuclei
		GVBD	M II	
0	130	111 (83.7 ± 2.3)[a]	74 (55.8 ± 2.1)[a]	13 (8.7 ± 2.0)
0.1	138	124 (89.2 ± 2.8)[a,b]	79 (58.5 ± 4.1)[a]	12 (7.7 ± 2.8)
0.5	137	124 (90.5 ± 1.2)[a,b]	82 (59.8 ± 3.5)[a,b]	7 (6.5 ± 3.4)
1.0	133	125 (92.9 ± 3.0)[b]	89 (67.2 ± 3.4)[b]	9 (4.7 ± 1.8)
2.5	110	96 (88.0 ± 3.6)[a,b]	67 (61.0 ± 3.9)[a,b]	4 (3.7 ± 0.3)
5.0	115	96 (83.7 ± 1.7)[a]	51 (43.7 ± 1.7)[c]	4 (3.8 ± 0.7)

*All of the experiments were repeated 4-7 times. Data are expressed as the mean ± SEM.

** GVBD; germinal vesicle break down, M II; metaphase II.

[a-c] The values with different superscript letters in the same column are significantly different ($P < 0.05$).

Table 1.2. Effects of sericin supplementation in the maturation medium on the fertilization and development of porcine oocytes*

Concentration of sericin (%)	No. of oocytes examined	No. (%) of oocytes fertilized		No. of oocytes examined	No. (%) of embryos	
		Total	Monospermic**		Cleaved	Developed to blastocysts
0	117	67 (59.5 ± 2.5)^{a,b}	33 (48.5 ± 4.0)^{a}	257	155 (60.1 ± 3.3)^{a,b}	30 (11.3 ± 2.3)^{a,b}
0.1	127	66 (55.8 ± 4.6)^{b}	36 (53.0 ± 3.8)^{a,b}	254	146 (57.5 ± 3.7)^{a,b}	17 (7.1 ± 1.5)^{b,c}
0.5	126	73 (57.3 ± 3.5)^{a,b}	39 (51.6 ± 2.8)^{a,b}	259	150 (57.6 ± 5.0)^{a,b}	28 (11.7 ± 1.7)^{a,b}
1.0	110	72 (67.2 ± 3.9)^{a}	40 (53.3 ± 2.7)^{a,b}	241	159 (66.3 ± 4.0)^{a,b}	33 (14.5 ± 1.8)^{a}
2.5	113	61 (53.5 ± 5.7)^{b,c}	34 (55.4 ± 6.1)^{a,b}	260	184 (69.7 ± 5.6)^{a}	28 (10.2 ± 1.4)^{a,c}
5.0	112	46 (41.0 ± 1.4)^{c}	29 (62.2 ± 2.8)^{b}	248	140 (54.4 ± 5.5)^{b}	14 (4.8 ± 1.7)^{c}

*All of the experiments were repeated 4-6 times. Data are expressed as the mean ± SEM.

**The proportions of monospermic fertilization were calculated by dividing the numbers of monospermic fertilized oocytes by the total number of fertilized oocytes.

$^{a-c}$ The values with different superscript letters in the same column are significantly different ($P < 0.05$).

Figure 1.1. Effects of sericin supplementation in the maturation medium on the total cell number (a) and DNA fragmentation index (b) of porcine blastocysts (mean SEM). All of the blastocysts (14–33 embryos) developed after IVF were used to estimate the cell number and number of DNA-fragmented nuclei. The bars with different letters are significantly different ($P < 0.05$)

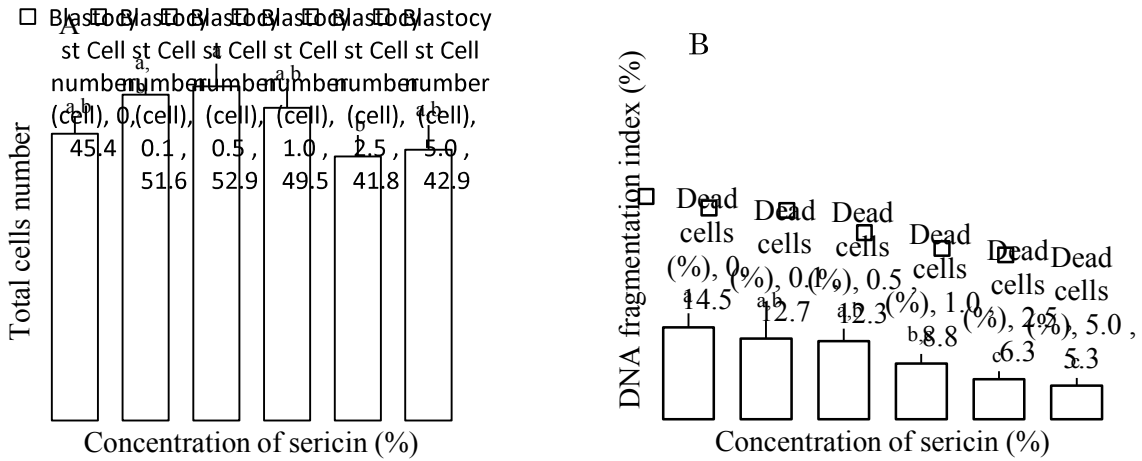


Table 2.1. Effect of melatonin supplementation during embryo culture on the development and quality of *in vitro* fertilized porcine embryos

Concentration of melatonin (ng/ml)	Number of examined zygotes	Number (%)* of embryos		DNA-fragmented nuclei (%)* in blastocyst
		Cleaved	Developed to blastocyst	
0	276	201 (73.2 ± 3.0)	12 (4.2 ± 2.0)[a]	7.2 ±2.5[a]
10	288	198 (69.2 ± 4.2)	13 (4.3 ± 1.5)[a]	3.2 ±1.0[a,b]
25	284	217 (76.4 ± 3.2)	30 (10.7 ± 2.7)[b]	5.1 ±1.1[a,b]
50	295	213 (72.5 ± 5.4)	21 (7.1 ± 2.0)[a,b]	2.1 ±0.5[b]

Six replicated trials were carried out.
*Percentages are expressed as the mean SEM.
a,bValues with different superscript letters in the same column differ significantly ($P < 0.05$).

Table 2.2. Effect of melatonin supplementation during maturation culture on the development and quality of porcine oocytes after *in vitro* fertilization

Concentration of melatonin (ng/ml)	Number of examined oocytes	Number (%)* of embryos		DNA-fragmented nuclei (%)* in blastocyst
		Cleaved	Developed to blastocyst	
0	295	222 (74.2 ± 5.0)	31 (11.1 ± 2.8)	8.9 ± 1.8^{b}
10	327	266 (81.3 ± 3.2)	36 (11.0 ± 2.9)	$5.2 \pm 0.9^{a,c}$
25	329	284 (86.4 ± 2.0)	50 (15.0 ± 4.2)	2.7 ± 0.6^{a}
50	338	283 (83.9 ± 2.5)	50 (14.7 ± 2.1)	5.4 ± 0.8^{c}

Seven replicated trials were carried out.
*Percentages are expressed as the mean SEM.
a,b,cValues with different superscript letters in the same column differ significantly ($P < 0.05$).

Table 3.1. The effects of astaxanthin supplementation on the maturation and fertilization rates of porcine oocytes exposed to 41.0 °C during maturation

Exposure temperature (°C)	Astaxanthin (ppm)	Number of oocytes	Number of oocytes that reached MII (maturation rate; % (mean± SEM)	Number of oocytes	Number of fertilized oocytes (fertilization rate (%) (mean± SEM)*	
					Total	Monospermic
38.5 (control temperature)	0	86	39 (47.9 ± 8.3)^{c}	105	35 (33.3 ± 6.7)^{c}	19 (61.1 ± 12.3)^{ab}
	0.25	106	56 (54.5 ± 5.5)^{bc}	106	53 (50.8 ± 7.1)^{ab}	32 (61.3 ± 6.2)^{a}
	0.5	100	66 (69.4 ± 3.5)^{a}	108	62 (56.8 ± 6.0)^{ab}	42 (67.7 ± 3.9)^{a}
	1.0	98	67 (67.0 ± 4.3)^{ab}	106	59 (57.1 ± 5.7)^{a}	43 (73.0 ± 6.5)^{ab}
41.0 (heat stress)	0	108	20 (16.5 ± 2.8)^{d}	107	23 (25.2 ± 3.9)^{c}	18 (83.3 ± 7.0)^{b}
	0.25	107	27 (28.1 ± 2.5)^{d}	102	42 (40.2 ± 5.0)^{bc}	25 (49.5 ± 8.2)^{a}
	0.5	96	48 (52.4 ± 5.5)^{c}	104	54 (54.9 ± 5.9) ^{ab}	38 (73.4 ± 4.6)^{ab}
	1.0	109	50 (50.5 ± 6.6)^{c}	105	46 (44.8 ± 3.8)^{ac}	30 (67.2 ± 5.8)^{a}

n=6 (maturation rate) or 9 (fertilization rate) independent experiments. MII: metaphase II
$^{a-d}$ Values with different superscripts in the same column differ significantly ($P < 0.05$)

*The monospermic fertilization rate was defined as a ratio of the number of monospermic oocytes and the total number of fertilized oocytes.

Table 3.2. The effects of astaxanthin supplementation on the development and quality of porcine Day 8 blastocyst derived from oocytes exposed to 41.0 °C during maturation (mean±SEM)

Exposure temperature (°C)	Astaxanthin (ppm)	Number of oocytes	Number (%) of embryos		Number of embryos	Total cell number	Apoptotic nucleus index*
			Cleaved (cleavage rate)	Developed to blastocysts (blastocyst formation rate)			
38.5 (control temperature)	0	235	160 (68.1 ± 4.7)^{B}	24 (10.7 ± 2.3)^{b}	21	42.4 ± 4.0^{a}	6.1 ± 1.1^{ab}
	0.25	227	178 (79.5 ± 3.2)^{A}	48 (20.0 ± 2.3)^{a}	33	43.1 ± 2.9^{a}	5.0 ± 0.8^{ab}
	0.5	226	155 (68.6 ± 2.9)^{B}	45 (20.2 ± 2. 9)^{a}	29	41.3 ± 2.4^{a}	3.9 ± 0.6^{b}
	1.0	240	186 (77.4 ± 3.6)^{AB}	42 (17.8 ± 1.7)^{a}	28	47.0 ± 4.3^{a}	5.0 ± 0.6^{ab}
41.0 (heat stress)	0	248	154 (62.5 ± 2.6)^{B}	2 (0.9 ± 0.6)^{c}	2	40.0 ± 8.0^{a}	4.5 ± 1.5^{ab}
	0.25	251	149 (58.1 ± 3.5)^{B}	16 (6.4 ± 1.4)^{b}	16	47.7 ± 7.3^{a}	7.1 ± 1.1^{a}
	0.5	232	168 (72.7 ± 4.3)^{A}	23 (9.8 ± 1.2)^{b}	15	42.9 ± 6.4^{a}	4.3 ± 0.9^{ab}
	1.0	222	149 (60.7 ± 4.3)^{B}	22 (10.0 ± 1.3)^{b}	13	38.1 ± 5.4^{a}	6.5 ± 1.0^{ab}

^{abc} Values with different superscripts in the same column differ significantly ($P < 0.05$)

^{AB} Values with different superscripts differ significantly within the same temperature ($P < 0.05$) **-** a significant interaction between temperature exposure × astaxanthin concentration was noted for the cleavage rate ($P < 0.01$).

***** The apoptotic index was defined as the ratio of the number of cells containing apoptotic nucleus and the total number of cells in a blastocyst.

n=7 independent experiments.

Figure3.1. Representative images of developmental stage of porcine oocytes stained by Hoechst 33342. (A) germinal vesicle, (B) germinal vesicle break down, (C) metaphase I, (D) anaphase I, (E) metaphase II with a polar body (arrow) and (F) degenerated oocyte. Scale bar = 50 μm.

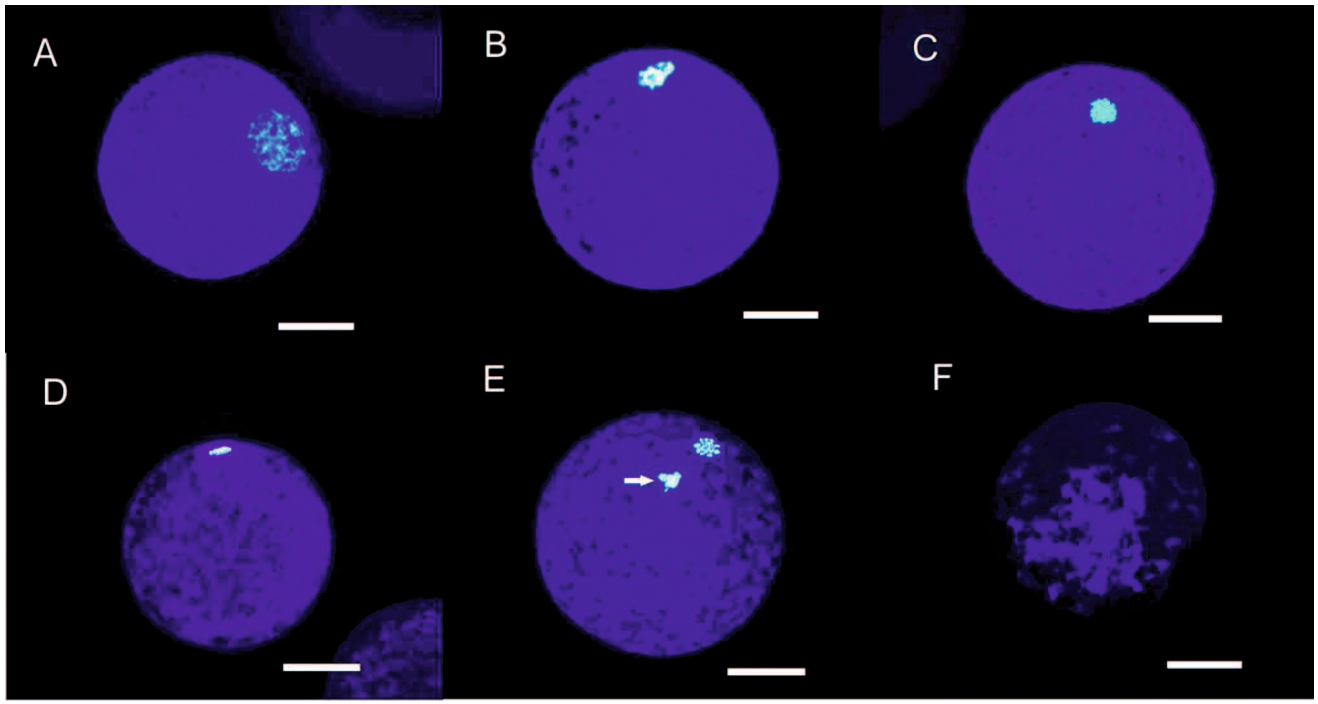


Figure 3.2. Representative images of porcine fertilized oocytes stained by aceto-orcein. (A) Two pronuclei (FPN - female pronucleus, MPN - male pronucleus) and two polar bodies (arrows) were observed in monospermic oocyte. (B) Three pronuclei (arrows) were observed in polyspermic oocyte. Scale bar = 50 μm.

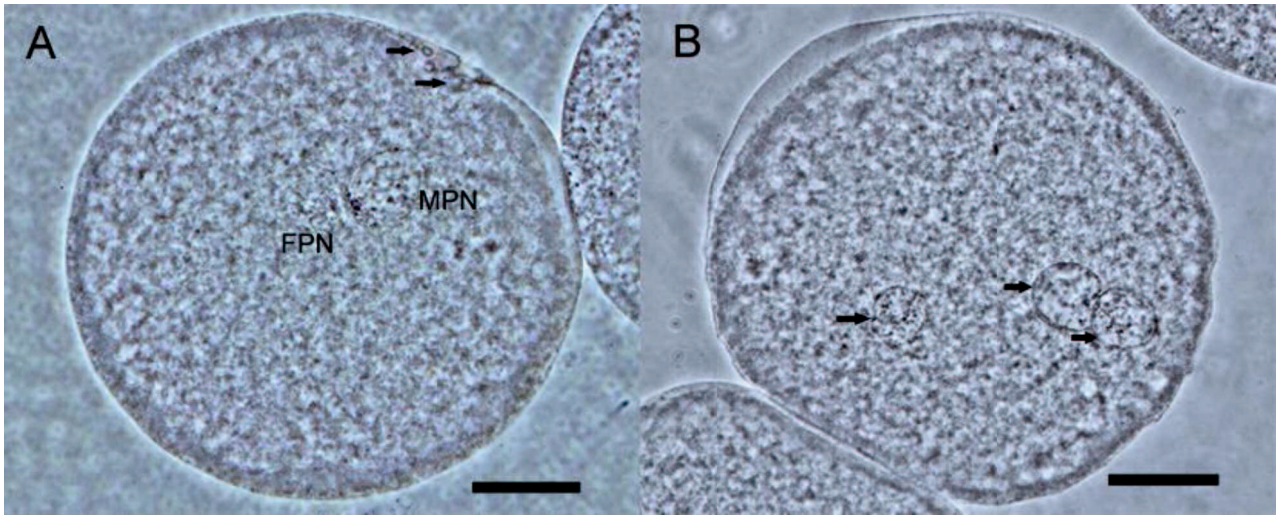


Figure 3.3. Representative images of porcine blastocysts subjected to DAPI (A) and TUNEL (B). Scale bar = 50 μm. Blue and green colors represent regular nuclei and apoptotic nuclei**,** respectively.

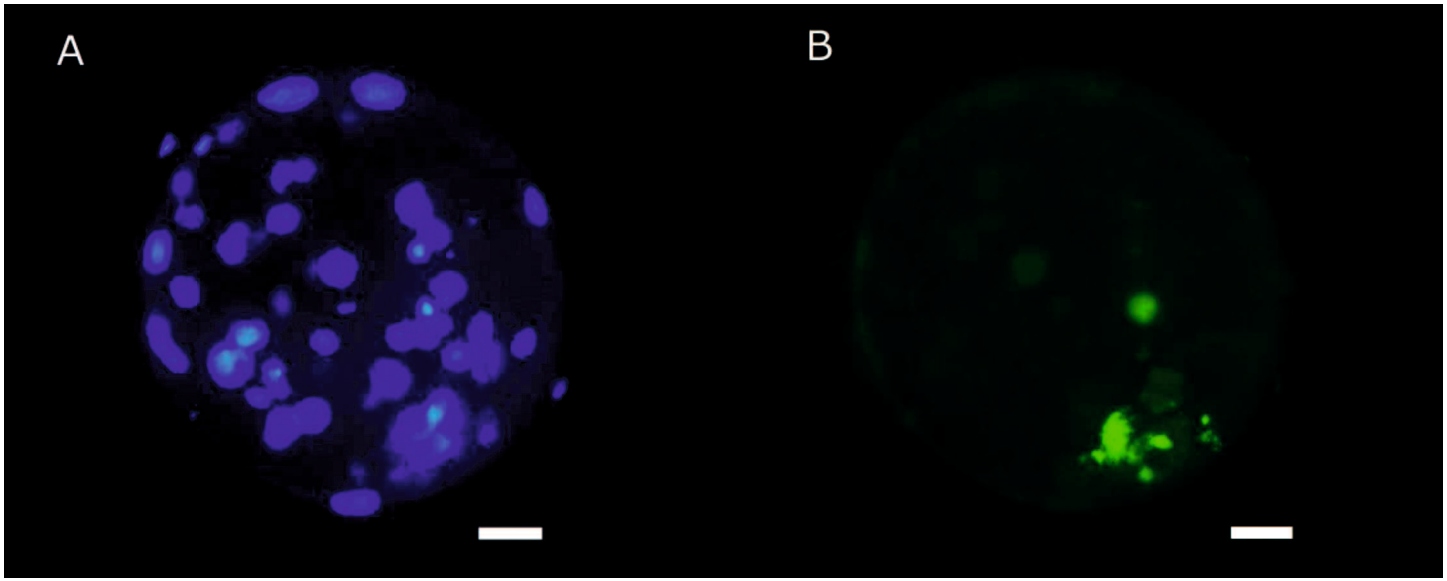
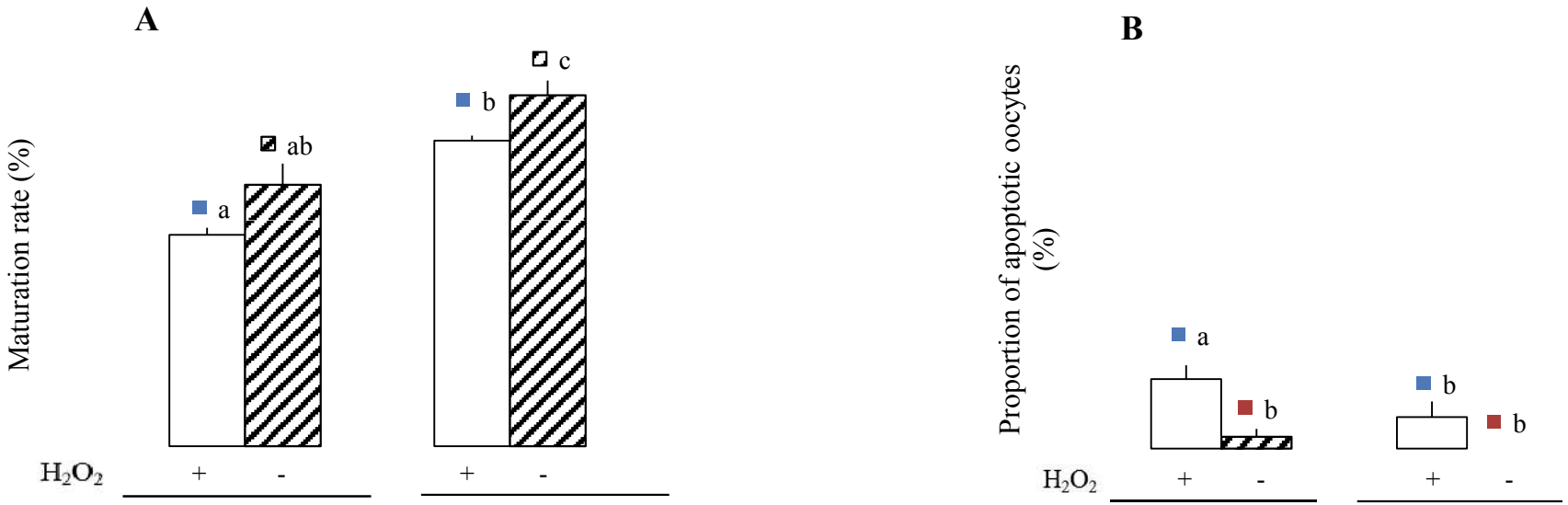


Figure 3.4. The effect of astaxanthin (0.5 ppm) supplementation on the maturation rate (A) and proportion of oocytes with apoptotic nucleus (B) of porcine oocytes exposed to 1.0 mM H2O2 during *in vitro* maturation. Oocytes cultured without astaxanthin served as controls. Each bar represents the mean value W SEM (n = 4 replicates, each with 96–102 oocytes per treatment). Bars with different letters differ significantly ($P < 0.05$).



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